EFFECTS OF SODIUM HYALURONATE AND TRIAMCINOLONE ACETONIDE ON PROTEOGLYCAN METABOLISM IN EQUINE ARTICULAR CHONDROCYTES TREATED WITH INTERLEUKIN-1

BY

ELYSIA CHRISTINA SCHAEFER

THESIS

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Advisor:

Assistant Professor Allison Stewart

ABSTRACT

The objective of this study is to determine if the effects of a high molecular weight sodium hyaluronate (HA) alone or in combination with triamcinolone acetate (TA) can mitigate chondrocyte proteoglycan catabolism caused by interleukin-1 (IL-1) administration. Chondrocytes were collected from fetlock joints of ten horses euthanized for reasons unrelated to joint disease. Chondrocyte pellets were treated with media (negative control); media containing IL-1 only (positive control); or media containing IL-1 with HA only (0.5 or 2.0 mg/mL), TA only (0.06 or 0.6 mg/mL), or HA (0.5 or 2.0 mg/mL) and TA (0.06 or 0.6 mg/mL) in combination. Chondrocyte pellets were assayed for newly synthesized GAG, total GAG content, total DNA content, and mRNA levels of collagen type II, aggrecan, and COX-2. The high concentration of HA (2.0 mg/mL) increased GAG synthesis while the high concentration of TA (0.6 mg/mL) decreased loss of GAG into the media. Both the high concentration of HA and TA increased the total GAG content within the pellet. There was no change in pellet DNA content with either treatment. TA reduced COX-2 mRNA levels as well as aggrecan and collagen type II expression. Treatment with HA had no effect on mRNA levels of COX-2, aggrecan or collagen type II. These results indicate that the high concentration of HA or TA alone or in combination will mitigate effects of IL-1 administration on proteoglycan catabolism of equine articular chondrocytes.

To my parents

For always believing in me and encouraging me to reach for the stars

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1. INTRODUCTION

The horse industry generates an average of $39 billion annually in the United States and has a total impact of $101.5 billion on the United States Gross Domestic Product (Deloitte Consulting LLP, 2005). There are approximately 9.2 million horses in the United States with 81% involved in recreation, showing, or racing (Deloitte Consulting, 2005). The major cause of wastage in equine athletes is due to lameness associated with joint pain and osteoarthritis (Pool and Meager, 1990; Rossdale, et al., 1985; Jeffcott, et al. 1982; and Stover, 2003). Current therapies utilized in the treatment of lameness associated with osteoarthritis include oral supplements such as glucosamine and chondroitin sulfate, oral medications such as non-steroidal anti-inflammatories, and intra-articular medications such as corticosteroids, hyaluronic acid, polysulfated glycosaminoglycans, and interleukin-1 receptor antagonist protein (IRAP) (Goodrich, et al., 2006; Frisbie, et al., 2002). While current clinical evidence suggests combination therapy with intra-articular corticosteroids and hyaluronic acid may be most beneficial, little scientific evidence has been published in this area. The purpose of this study was to evaluate two commonly used intra-articular therapies, corticosteroids and hyaluronic acid (HA), in combination for treatment of osteoarthritis.

Intra-articular injections of corticosteroids rapidly resolve joint inflammation, synovitis, and pain (Frisbie, et al., 1998; Shoemaker, et al., 1992; Murphy, et al., 2000; and Frisbie, et al., 1997). Corticosteroids remain the gold standard for keeping horses with osteoarthritis in work while alleviating their pain (Frisbie, et al., 1997). The anti-inflammatory mechanisms of corticosteroids are two-fold. Corticosteroids suppress arachidonic acid metabolism through lipocortin-induced phospholipase inhibition

(Gilron, 2004). This inhibition helps to stabilize the phospholipids within the cell membrane, making them unavailable for entrance into the arachidonic cascade. Corticosteroids also block production of pro-inflammatory cytokines, such as interleukin-1 (IL-1), by binding to cytoplasmic receptors and modulating gene transcription (Gilron, 2004). Two of the more common corticosteroids frequently used are methylprednisolone acetate (MPA) and triamcinolone acetate (TA). Previous studies have shown higher doses of MPA can have detrimental effects both on normal and abnormal articular cartilage by impairing chondrocyte activity, inhibiting glycosaminoglycan (GAG) and proteoglycan synthesis within the articular cartilage matrix, and decreasing mRNA expression of collagen type II (Doyle, et al., 2005; Yates, et al., 2006; Frisbie, et al., 1998; Shoemaker, et al., 1992; and Murphy, et al., 2000). In contrast, studies on TA have shown many beneficial effects on abnormal articular cartilage. In these studies, TA decreased IL-1 induced GAG degradation, increased proteoglycan synthesis, and protected chondrocyte viability (Frisbie, et al., 1997; Sandler, et al., 2004; and Dechant, et al., 2003). Based on many studies, 6 to 18 mg of intra-articular TA is recommended for treatment in high motion joints (Carson, 2005).

Clinically, HA has been used as a therapeutic treatment for osteoarthritis. In a normal joint, HA is secreted into the synovial fluid by joint capsule synoviocytes and serves as boundary lubrication (Schmidt, et al., 2007). It is also an important component of the articular cartilage where it binds to chondrocyte CD44 receptors and serves as a backbone of attachment sites for proteoglycan structures (Akmal, et al., 2005; Goodrich and Nixon, 2006). Proteoglycans maintain the hydrostatic pressure of cartilage allowing resistance to compressive forces during weight bearing and is depleted first in the early

stages of osteoarthritis (Rydell, et al., 1970; Young, et al., 2006). Previously documented beneficial effects of HA are increasing chondrocyte metabolism, increasing the GAG content within the cartilage matrix, and decreasing the activity of pro-inflammatory mediators resulting in decreased matrix degradation (Yates, et al., 2006; Akmal, et al., 2005; Rydell, et al., 1970; Iwata, 1993; and Moreland, 2003). However, it remains uncertain if the molecular weight of various HA products plays a significant factor in the effectiveness of these functions. Several studies have demonstrated that a high molecular weight HA may have a longer efficacy and increased metabolic and anti-inflammatory properties when compared to a low molecular weight HA (White, et al., 1999; McIlwraith, 1996; Phillips, 1980; and Cheney, 1996). However, other studies claim little to no difference between high and low molecular weight HA products (Aviad and Houpt, 1994; Aviad, et al., 1988).

Two recently published studies have evaluated combination therapy with MPA and HA both on normal equine cartilage explants and an IL-1-induced inflammatory model of chondrocyte metabolism (Doyle, et al., 2005; Yates, et al., 2006). The cartilage explant study demonstrated detrimental effects of MPA on normal articular cartilage and showed addition of a medium-molecular weight HA had little effect on the corticosteroid-induced proteoglycan catabolism of the cartilage matrix (Doyle, et al., 2005). The later study showed beneficial effects on proteoglycan metabolism by using lower doses of MPA and a medium-molecular weight HA on inflamed (IL-1 treated) chondrocyte pellets (Yates, et al., 2006). The purpose of this study was to evaluate the effects of two of the most commonly used intra-articular therapies in high motion joints. This included a medium-acting corticosteroid (TA) and a high molecular weight HA. Our hypothesis

was that administration of HA alone or in combination with TA would mitigate the chondrocyte proteoglycan catabolism caused by IL-1 administration.

CHAPTER 2. LITERATURE REVIEW

2.1 Structure and Function of Healthy Synovial Joints

Synovial, or diarthrodial, joints serve two distinct functions in the body. First, they allow movement. Second, they allow the transfer of load between bones. Synovial joints consist of a joint capsule lined with synovium, synovial fluid, articular cartilage and its associated extracellular matrix. Each of these components plays a crucial role in joint homeostasis to allow proper functioning of the joint through movement and load transfer.

2.1.1 Synovium

The inner surface of the joint capsule is lined with a synovial membrane. This synovium consists of vascularized connective tissue that lacks a basement membrane (Todhunter, 1996b; Henderson, et al., 1985). The blood supply to the synovium branches from the joint capsule and extend into the subsynovium, within 5 to 10μm of the intimal surface (Todhunter, 1996b). These vessels are also accompanied by lymphatics and nerves. The predominant cell type in the synovium consists of synoviocytes. There are several different types of synoviocytes: type A, type B, and type C (Saw, 2007; Frisbie, 2006; Todhunter, 1996b; Iwanaga, et al., 2000; Gerwin, et al., 2006; Burmester, et al., 1983; Sutton, et al., 2009). Type A synoviocytes are important in phagocytosis (Burmester, et al., 1983; Sutton, et al., 2009). Type B synoviocytes are important in secretory functions and are the main producers of synovial fluid for the joint (Iwanaga, et al., 2000). In particular, these cells produce and secrete hyaluronan which serves as a key component in boundary lubrication and load transfer (Todhunter, 1996b; Sutton, et al.,

2009). Lubricin, another key component of boundary lubrication, is also synthesized by the synovium (Todhunter, 1996b; Saw, et al., 2007; Schmidt, et al., 2007a). Less information is known about the third type of synoviocytes, type C, which consist of intermediate or undifferentiated cells that may serve a reparative role in synovium (Saw, 2007; Frisbie, 2006; Todhunter, 1996b; Gerwin, et al., 2006; Sutton, et al., 2009).

2.1.2 Synovial Fluid

Synovial fluid, produced by the synovium, is an ultrafiltrate of blood plasma that also contains a high concentration of hyaluronan (Gerwin, et al., 2006; Todhunter, 1996b; Frisbie, 2006; Walsh, et al., 1997; Goodrich, et al. 2006). In addition, other molecules such as proteinase, collagenases, and prostaglandins, and cell types such as monocytes, lymphocytes and other polymorphonuclear leukocytes, are present (Todhunter, 1996b). However, normal equine synovial fluid contains less than 500 nucleated cells/μL (Todhunter, 1996b). Due to the absence of an intimal basement membrane and the close proximity of capillaries to the synovium, fluid exchange between plasma and synovial fluid can occur. This facilitates the exchange of solutes governed by Starling's forces (hydrostatic and oncotic pressure differences between the plasma and synovial fluid) (Levick, 1984).

The synovial fluid provides nutrients to the avascular articular cartilage and also serves as joint lubrication (Saw, et al., 2007; Gerwin, et al., 2006; Frisbie, 2006; Aigner, et al., 2006; Sutton, et al., 2009). The increased viscosity of the synovial fluid is attributed to the high concentration of hyaluronan (Gerwin, et al., 2006; Todhunter, 1996b; Hadler, et al., 1977). Normal equine synovial fluid contains roughly 0.5 mg/mL

hyaluronan (Saari, et al., 1989). The viscous nature of the synovial fluid allows it to provide joint lubrication to the synovial membrane and the articular cartilage through two different mechanisms: fluid film lubrication and boundary lubrication (Saw, et al., 2007; Davis, et al., 1978; Ateshian, et al., 2005; Schmidt, et al., 2007b; Frisbie, 2006). Fluid film lubrication involves pressurized fluid separating the joint surfaces. This occurs when a load is applied and interstitial fluid becomes pressurized causing leakage out of the extracellular matrix to form a fluid film between the opposing joint surfaces. The viscosity of this fluid increases to an osmotic pressure equivalent to the pressure being applied by the load (Todhunter, 1996b; Saw, et al., 2007; Davis, et al., 1978). Boundary lubrication involves a layer of lubrication along both articular surfaces that prevents direct surface to surface contact (Saw, et al., 2007; Schmidt, et al., 2009b). Lubricin, a glycoprotein, is the key lubricating component in synovial fluid and works in conjunction with hyaluronic acid to decrease resistance to motion along the articular cartilage (Gerwin, et al., 2006; Saw, et al., 2007; Swann, et al., 1985). Hyaluronic acid is the major component responsible for lubrication of the synovial membrane surface (Davis, et al., 1978). Boundary lubrication is critical to the protection and maintenance of joint surfaces to mitigate normal wear (Schmidt, et al., 2007b).

2.1.3 Articular Cartilage

Articular cartilage is the weight-bearing component of the synovial joint. It is avascular, aneural, and alymphatic (Dijkgraaf, et al., 1995; Saw, et al, 2007, Frisbie, 2006; Aigner, et al., 2006). Nutrition and elimination of waste products are dependent on diffusion through the extracellular matrix and the synovial membrane (Frisbie, 2006;

Dijkgraaf, et al., 1995; Gerwin, et al., 2006). Pain perception and proprioception are dependent on the nerve endings in the synovial membrane, joint capsule and underlying subchondral bone (Dijkgraaf, et al., 1995; Todhunter, 1996b). Articular cartilage is a connective tissue composed of chondrocytes and a specialized extracellular matrix (Frisbie, 2006; Gerwin, et al., 2006; Dijkgraaf, et al., 1995; Saw, et al., 2007).

Articular cartilage is arranged into the superficial zone, the transitional zone, the radial zone, the tidemark, and the calcified zone (Todhunter, 1996b; Saw, et al., 2007; Frisbie, 2006; Aigner, et al., 2006). The superficial zone (or tangential zone) has the highest cell density, with small and flat chondrocytes oriented parallel to the articular surface (Todhunter, 1996b). In the superficial zone, collagen fibrils are tangential to the surface (Frisbie, 2006). The transitional zone contains large and more rounded chondrocytes when compared to the superficial zone (Todhunter, 1996b; Frisbie, 2006). In the transitional zone, collagen fibrils are arranged in a complex 3-dimensional fashion with longer fibrils oriented somewhat perpendicular to the surface and shorter fibrils branching off the longer ones (Todhunter, 1996b; Clark, 1985). The radial zone chondrocytes are larger and oriented perpendicular to the articular surface (Saw, et al., 2007; Frisbie, 2006). In the radial zone, collagen fibrils are larger and positioned predominantly perpendicular to the articular surface (Todhunter, 1996b). The collagen meshwork is more rigid in the radial zone than in the transitional zone. The tidemark is the junction between the noncalcified cartilage and calcified cartilage observed on histological sections (Frisbie, 2006). The calcified zone contains the cement line formed during the endochondral ossification of the growth plate. The collagen fibrils are

arranged perpendicular to the articular surface and create a rigid meshwork interspersed with hydroxyapatite crystals (Todhunter, 2006b).

2.1.3.1 Chondrocytes

The major cellular component of articular cartilage is the chondrocyte. Chondrocytes compose anywhere from 1 to 12% of the articular cartilage makeup (Todhunter, 1996b). Nutrition is provided from the synovial fluid as load compression forces affect movement of interstitial fluid in and out of the extracellular matrix carrying nutrition towards the chondrocytes and transporting waste products away towards the synovial fluid (Gerwin, et al., 2006; Frisbie, 2006; Dijkgraaf, et al., 1995). While they constitute only a very small fraction of the articular cartilage volume, they are responsible for production and maintenance of the entire extracellular matrix, including collagen, proteoglycans and numerous enzymes for cartilage metabolism (Todhunter, 1996b; Saw, et al., 2007). In addition, chondrocytes also have control over the location of adjacent extracellular components through specific cell membrane receptors (Dijkgraaf, et al., 1995; Frisbie, 2006). These cell receptors are integral membrane proteins that contain extracellular glycosaminoglycan chains capable of binding collagen and fibronectin in the extracellular matrix (Dijkgraaf, et al., 1995).

2.1.3.2 Extracellular Matrix

The extracellular matrix of articular hyaline cartilage consists of water, collagen, proteoglycans, structural glycoproteins, and small amounts of lipids and inorganic components (Dijkgraaf, et al., 1995). The high water content (70 to 80%) of the hyaline

cartilage extracellular matrix is responsible for the translucent, glassy appearance (Todhunter, 1996b). The majority of the dry weight of articular cartilage is due to the collagen (50-60%) and proteoglycan (20-40%) volume, with the remainder of the dry weight constituting glycoproteins (10%), minerals (3%), and lipids (1%) (Todhunter, 1996b).

The majority of the collagen in articular cartilage is type II (85-95% of the total) (Saw, et al., 2007; Mayne, et al., 1986; Eyre, et al., 1991; Vachon, et al., 1990). The collagen fibrils are arranged in bundles, creating a meshwork kept together by cross-links with collagen type IX and anchoring proteins such as fibronectin (Howell, et al., 1992; Dean, 1991). This network provides the tensile strength and shape of articular cartilage and counteracts the swelling pressure of the hydrophilic proteoglycans within the extracellular matrix (Maroudas, 1979; Roughly, et al., 1994; Frisbie, 2006).

Proteoglycans are the other major solid component of the extracellular matrix. They can be divided into two broad categories including large aggregating proteoglycans (aggrecan) and small nonaggregating proteoglycans (decorin, biglycan, and fibromodulin) (Platt, 1996; Frisbie, 2006). These structures are complex macromolecules that consist of a core protein covalently linked to glycosaminoglycan chains of varying lengths and bound to hyaluronan via a link protein (Hamerman, et al., 1985; Dijkgraaf, et al., 1995; Frisbie, 2006). The major glycosaminoglycans within the extracellular matrix are chondroitin 4-sulfate, chondroitin 6-sulfate, keratan sulfate, and hyaluronan. These glycosaminoglycans constitute about 90% of the glycosaminoglycan side chains (Hamerman, et al., 1985; Howell, 1989; Platt, 1996; Dijkgraaf, et al., 1995). Other smaller glycosaminoglycan side chains found within the extracellular matrix include

dermatan sulfates (biglycan and decorin) and fibromodulin (Todhunter, 1996b; Platt, 1996; Hedlund, et al., 1994; Wiberg, et al., 2001). These proteoglycan structures are intertwined within the collagen network, and due to their high water-binding capacity, also aid in providing the cartilage with its resilience, elasticity, sheer strength and self-lubrication (Aigner, et al., 2006; Dijkgraaf, et al., 1995).

Hyaluronan has a unique glycosaminoglycan structure. It is not attached to a core protein during synthesis and it is the only glycosaminoglycan that is not sulfated (Todhunter, 1996b). In articular cartilage, the molecular weight of hyaluronan varies from 3×10^2 to 1×10^6 kDa (Laurent, et al., 1986; Todhunter, 1996b). A large portion of the hyaluronan found in articular cartilage is associated with aggrecan, forming larger proteoglycan structures (Todhunter, 1996b; Roughley, et al., 1994). In addition to binding aggrecan, hyaluronan also binds to CD44 receptors on chondrocyte cell membranes and to other molecules in the extracellular matrix (Todhunter, 1996b; Ishida, et al., 1997).

Aggrecan is a core protein that contains chondroitin sulfate and keratan sulfate glycosaminoglycan side chains (Hascall, et al., 1974; Todhunter, 1996b). It is most often found bound in large aggregates to hyaluronan (Hascall, et al., 1974; Todhunter, 1996b; Lohmander, 1988; Frisbie, 2006). As many as 100 aggrecan monomers can be bound to a single hyaluronan chain by link proteins (Lohmander, 1988; Akeson, 2003). These link proteins simultaneously bind aggrecan and hyaluronan to make the dissociation of the two monomers difficult under physiologic conditions (Akeson, 2003; Franzen, et al., 1981; Hardingham, et al., 1979; Perin, et al., 1987).

The small nonaggregating proteoglycans can be subdivided into two groups: chondroitin sulfate-dermatan sulfate proteoglycans and keratan sulfate proteoglycans (Todhunter, 1996b). Heparan sulfate proteoglycans have been identified on the chondrocyte cell surface, but have yet to be identified in the extracellular matrix (Todhunter, 1996b). Three small nonaggregating proteoglycans have been identified within the extracellular matrix of the articular cartilage: biglycan, decorin, and fibromodulin (Platt, 1996; Rosenberg, 1992; Hedlund, et al., 1994; Wiberg, et al., 2001). Little is understood about the *in vivo* function of these small proteoglycans, but *in vitro* studies suggest that they may play a role in collagen fibril size, and the multiplication, differentiation, and migration of cells (Todhunter, 1996b; Hedbom, et al., 1989; Rosenberg 1992).

The structural glycoproteins are noncollagenous and nonproteoglycan structures whose functions are relatively unknown, except for the link proteins (Fife, et al., 1993; Todhunter, 1996b). In general, the structural glycoproteins interact with cellular receptors and regulate adhesion, migration, proliferation, and differentiation of the chondrocytes (Trelstad, 1989; Dean, et al., 2003). The two main structural glycoproteins are fibronectin and laminin. Fibronectin is a large, adhesive glycoprotein that aggregates near the chondrocyte cell membrane and in the extracellular matrix (Dijkgraaf, et al., 1995). Laminin is found predominantly in the basement membranes and as a receptor-bound component of the cell surface (Dijkgraaf, et al., 1995). Chondronectin has also been found to have adhesive properties, binding chondrocytes to type II collagen fibrils (Hewitt, et al., 1982).

2.1.3.3 Chondrocyte Metabolism and Extracellular Matrix Turnover

Chondrocytes account for a small volume of the articular cartilage. However, they are responsible for the synthesis and degradation of all extracellular matrix components (Dijkgraaf, et al., 1995; Todhunter, 1996b). Collagen begins translation in the rough endoplasmic reticulum of the chondrocyte where it starts as a procollagen molecule that subsequently undergoes posttranslational hydroxylation and glycosylation. The hydroxylase enzymes that act as a catalyst for the hydroxylation are kept in an active form by ascorbic acid (Pacifici, et al., 1988; Todhunter, 1996b). The collagen molecules are then woven into a triple helix structure in the Golgi apparatus and secreted into the extracellular matrix. The N- and C-terminal propeptides are then cleaved and the fibrils are crosslinked (Leblond, 1989; Todhunter, 1996b).

Proteoglycans are also synthesized by the chondrocytes. The core protein is the first part to be synthesized within the rough endoplasmic reticulum. It then undergoes addition of glycosaminoglycan chains in the Golgi apparatus, followed by sulfation of these chains (Lohmander, et al., 1986; Platt, 1996). Because sulfation is a late stage in the synthesis of the proteoglycan molecules, it is a convenient method of monitoring proteoglycan synthesis by radioactive labeling with $^{35}SO_4$ (Todhunter, 1996b). Hyaluronan lacks a protein core and is therefore synthesized by a different route. Its synthesis occurs on the inner plasma membrane before extruding into the extracellular space (Prehm, 1983; Prehm, 1984). Because it does not undergo synthesis in the Golgi apparatus, hylauronan is not sulfated (Todhunter, 1996b).

Chondrocytes are also responsible for maintenance, organization and regulation of the extracellular matrix. They receive chemical and hormonal stimuli from the synovial

fluid that diffuses into the extracellular matrix to interact with the chondrocytes (Todhunter, 1996b). In response, they secrete and regulate proteases, cytokines, and growth factors to maintain this homeostasis (Dijkgraaf, et al., 1995; Platt, 1996; Frisbie 2006). The molecules that may diffuse into the cartilage from the synovial fluid are regulated by the proteoglycan concentration within the extracellular matrix; larger molecules being unable to diffuse through high concentrations of proteoglycan (Maroudas, 1970; Maroudas, 1976; Maroudas, et al., 1986). Cytokines and growth factors are polypeptides that influence the growth, differentiation and metabolic activity of the chondrocytes (Howell, et al., 1992; Dijkgraaf, et al., 1995). In general, cytokines act in a catabolic fashion and growth factors act in an anabolic fashion in the articular cartilage. Cytokines, such as interleukins and tumor necrosis factor α, can be synthesized by chondrocytes, synovium or inflammatory cells and act to stimulate proteoglycan depletion (Dijkgraaf, et al., 1995; Todhunter, 1996b). They act primarily at the cell surface receptors and the signal is then relayed to the nucleus and results in transcription of factors resulting in decreased proteoglycan synthesis and up-regulation of proteases which result in increased proteoglycan degradation (Pelletier, et al., 1993; Howell, et al., 1992). Cytokine effects can also be negated by receptor antagonists such as interleukin-1 receptor antagonist (IL-1ra) and binding proteins such as tumor necrosis factor-binding protein (TNF-BP) (Pelletier, et al., 1991).

Growth factors can antagonize the catabolic effects of cytokines and stimulate proteoglycan synthesis (Tyler, et al., 1992; Dijkgraaf, et al., 1995). Several growth factors, such as insulin-like growth factor (IGF) and transforming growth factor (TGF), have been identified as having beneficial effects on proteoglycan synthesis in articular

cartilage (Tesch, et al., 1992; Roberts, et al., 1990). Both of these growth factors have been shown *in vitro* to increase the synthesis of proteoglycan and several extracellular matrix components (Morales, 1991; Morales, et al., 1988; Morales, 1993; Platt, et al., 1995; Platt, 1996). In addition, IGF and TGF act to negate the detrimental effects of certain cytokines by decreasing synthesis of certain proteinases and increasing synthesis of their corresponding inhibitors (Pelletier, et al., 1991; Dijkgraaf, et al., 1995; Morales, et al., 1988).

Interleukin-1 (IL-1) activity has been reported in normal equine synovial fluid and plays a major role in the homeostasis of the normal cartilage matrix (May, et al., 1992; Tyler, et al., 1992). *In vitro* studies have demonstrated its effects on upregulation of degradative enzymes (proteases) and other inflammatory cytokines (IL-6, IL-8, and TNF) (Tyler, et al., 1992; Dijkgraaf, et al., 1995; Platt, et al., 1994; Arner, et al., 1989; Gouze, et al., 2001). In addition, several studies have shown addition of IL-1 stimulating an upregulation of protease inhibitors such as tissue inhibitor of metalloprotease (TIMP) and plasminogen activator inhibitor (PAI) suggesting that IL-1 plays a more complex role in cartilage homeostasis and regulation of enzymatic activity (Sato, et al., 1990; Treadwell, et al., 1991).

IL-1 is a potent stimulator of metalloproteinase secretion by normal chondrocytes (Tetlow, et al., 2001; Dijkgraaf, et al., 1995; Bau, et al., 2002). Two of the more studied matrix metalloproteinases (MMP) that have been shown to have a significant effect on articular cartilage have been MMP-1 (collagenase) and MMP-3 (stromelysin) (Martel-Pelletier, 2004). Collagenase acts to break down the triple helix structure of the collagen fibrils, leaving the fragments susceptible to further proteolysis (Dijkgraaf, et al., 1995;

Gross, et al., 1980; Murphy, et al., 1993; Cawston, et al., 1999). Stromelysin has degradative effects on collagen as well as the proteoglycan components of the extracellular matrix including aggrecan and link proteins (Martel-Pelletier, et al., 1994; Bonassar, et al., 2005; Lark, et al., 1997; Nguyen, et al., 1989; Flannery, et al., 1992; Wu, et al., 1991). In normal articular cartilage, these MMPs are held in check by protease inhibitors like TIMP and PAI, which have been shown to be present at slightly higher concentrations than MMPs (Hamilton, et al., 1991; Dean, et al., 1989; Woessner, et al., 1991; Little, et al., 1999). Recently, another group of metalloproteinases have been identified that play a crucial role in aggrecan breakdown within the extracellular matrix (Tang, et al., 2001; Cal, et al., 2002). These are the aggrecanases, otherwise known as "A Disintegrin and Metalloproteinase with ThromboSpondin motif" (ADAMTS) (Huang, et al., 2008; Nagase, et al., 2003). It has been suggested that both ADAMTS-4 and ADAMTS-5 are constitutively expressed within normal synoviocytes and chondrocytes (Yamanishi, et al., 2002; Vankemmelbeke, et al., 2001). These ADAMTS are kept in check by TIMPs during normal cartilage homeostasis (Kashiwagi, et al., 2001; Hashimoto, et al., 2001).

2.2 The Pathophysiology of Osteoarthritis

Degeneration of the articular cartilage has been considered the "*sine qua non* of osteoarthritis" (McIlwraith, 1982). Three main concepts have been proposed as potential etiologies for osteoarthritis (Dijkgraaf, et al., 1995). The first concept is based on mechanical loading and biomaterial failure of the extracellular matrix and/or chondrocyte injury (Dijkgraaf, et al., 1995; Howell, 1989; Howell, et al., 1992; McIlwraith, 1996a).

This can occur through overloading (absolute or relative) or underloading of the cartilage. Absolute overloading occurs in normal articular cartilage when abnormal or repetitive mechanical loads exceed its adaptive capacity (Dijkgraaf, et al., 1995). Relative overloading occurs in abnormal articular cartilage when normal mechanical loads exceed the intrinsically reduced capacity of that cartilage (Dijkgraaf, et al., 1995). Underloading occurs when mechanical loads are below the normal physiologic range for that individual (Dijkgraaf, et al., 1995). The second concept involves failure of intrinsic chondrocyte-controlled remodeling and repair in response to insult (Dijkgraaf, et al., 1995; Howell, et al., 1992; McIlwraith, 1996a). A primary injury will disrupt the homeostatic balance of the extracellular matrix by the chondrocytes, resulting in accelerated extracellular matrix degradation (Dijkgraaf, et al., 1995; Howell, 1989). The third proposed concept involves extracartilagenous factors that may be the primary instigator, resulting in secondary cartilage changes (Dijkgraaf, et al., 1995; Howell, et al., 1992; McIlwraith, 1996a). These extracartilagenous factors may include subchondral bone microfractures, synovial responses such as changes in the synovial membrane or a reduction in the quality or quantity of synovial fluid, bony remodeling, and vascular changes (Dijkgraaf, et al., 1995, McIlwraith, 1996). Because osteoarthritis may have several different etiologies and manifest in several different ways, it has become necessary to classify the different types of osteoarthritis as type 1, type 2, and type 3 (McIlwraith, 1996a; Howell, et al., 1992). Type 1 is considered osteoarthritis "associated with synovitis and capsulitis" (McIlwraith, 1996a). Type 2 is considered osteoarthritis secondary to other problems such as intra-articular fractures, osteochondrosis, subchondral bone disease, and septic arthritis (McIlwraith, 1996a; Howell, et al., 1992). Type 3 is considered to be

osteoarthritis as “an incidental or nonprogressive articular cartilage erosion” (McIlwraith, 1996a).

Osteoarthritis has commonly been considered a degenerative condition of the articular cartilage, but the synovium may also play a role in the development and progression of the disease state (McIlwraith, 1996; Fell, et al., 1977; Smith, et al., 1997; Fiorito, et al., 2005; Sutton, et al., 2009). Trauma to the synovium occurs with repeated cyclic loading and results in the release of degradative enzymes, cytokines, and other inflammatory mediators (Evans, 1992; McIlwraith, 1982). In addition, the presence of IL-1 stimulates synthesis of such products as fibronectin and type I and type III collagen by synoviocytes, possibly attributing to the development of joint capsule fibrosis (Dijkgraaf, et al 1995; Haraoui, et al., 1991; Sledge, 1989). The presence of synovitis also causes an increase in intra-articular temperature which increases the rate of enzymatic activity, further accelerating the degradation process (Sledge, 1989). Joint effusion resulting from inflammation may also affect the intra-articular pressure (Dijkgraaf, et al., 1995). Normal pressure within most joints at rest is 1 to 2 mmHg subatmospheric (Stewart, 1999). Flexion of the joint increases the intra-articular pressure by as much as 3 mmHg (Stewart, 1999). However, in effusive joints, the resting intra-articular pressure may be slightly over atmospheric pressure (Stewart, 1999). With flexion during exercise, the atmospheric pressure may exceed 60 mmHg (Stewart, 1999). This increase in pressure may compromise the synovial blood flow and diffusion of nutrients through the articular cartilage to supply the chondrocytes, thereby impairing chondrocyte nutrition (Dijkgraaf, et al., 1995; McIlwraith, 1996a). This compromise in blood flow could also potentially lead to reperfusion injury as the joint moves through a

range of motion (Stewart, 1999; Levick, 1990; McIlwraith, 1996a; Merry, et al., 1991). Flexion of the effused joint would impair synovial blood flow leading to production of xanthine oxidase (Stewart, 1999; McIlwraith, 1996a; Allen, et al., 1989; Blake, et al., 1989). Upon relaxation of the joint, reperfusion would occur as well as the production of oxygen-derived free radicals leading to further chondrocyte and extracellular matrix damage (Stewart, 1999; McIlwraith, 1996a; Allen, et al., 1989).

In the horse, trauma is considered the most important initiating event in the development of osteoarthritis (McIlwraith, 1982; McIlwraith, 1996a; Goodrich, et al., 2006). Many factors may predispose the joints to trauma, including joint instability, fracture, faulty conformation, improper shoeing/imbalance, and type of work being performed (McIlwraith, 1982; McIlwraith, 1996a; Goodrich, et al., 2006). These factors lead to abnormal or repetitive biomechanical loading resulting in trauma to the articular cartilage and synovium (Goodrich, et al., 2006; McIlwraith, 1996a).

Progression of osteoarthritis can be divided into several stages, with each stage exhibiting increasing joint degradation (Howell, et al., 1989). The initial stage occurs after a primary insult and involves cartilage degradation with attempts at repair (Howell, et al., 1989; Dijkgraaf, et al., 1995). Histologically, this stage exhibits proliferation of chondrocytes as they increase their metabolic activity in an attempt to repair the damage inflicted upon the articular cartilage (Dijkgraaf, et al., 1995; de Bont, et al., 1985). Most importantly, there is a loss of proteoglycan from the extracellular matrix at this initial stage (McIlwraith, 1996a; Dijkgraaf, et al., 1995; Nelson, et al., 2006). Biochemically, there is an increase in synthesis of DNA and extracellular matrix components mediated by growth factors until a balance between synthesis and degradation has been reached

(Dijkgraaf, et al., 1995; Howell, et al., 1993). The next stage is reached when the degradation of the extracellular matrix by proteases has exceeded the synthesis by chondrocytes (Dijkgraaf, et al., 1995; Howell, et al., 1989). Histologically, this early stage is characterized by focal swelling of the articular cartilage, cartilage surface irregularities, disorganization of the collagen network, chondrocyte proliferation followed by necrosis, and uneven staining of proteoglycan in the extracellular matrix (Mankin, et al., 1989; de Bont, et al., 1985; de Bont, et al., 1985b). Biochemically, there is still increased synthesis of DNA, proteoglycans, collagen and structural glycoproteins (Dijkgraaf, et al., 1995; McIlwraith, 1996a). In addition, proteolytic enzyme synthesis and release is increased while protease inhibitor synthesis may be decreased (Dijkgraaf, et al., 1995; Yamada, et al., 1987). However, while the production of proteoglycan is increased, the composition of these newly synthesized proteoglycans is abnormal (Dijkgraaf, et al., 1995; Vasan, 1980). The glycosaminoglycan chains are shorter, the concentrations of various glycosaminoglycans are altered, and normal aggregation with hyaluronan is altered (Dijkgraaf, et al., 1995; Howell, 1989). The disruption of the collagen network allows increased water retention by the proteoglycans of the extracellular matrix and accounts for the swelling of the articular cartilage seen microscopically (Maroudas, 1976b; Sweet, et al., 1977; McIlwraith, 1996a; Aigner, et al., 2006; Goodrich, et al., 2006).

As the disease process progresses, further degradation of the articular cartilage is observed (Dijkgraaf, et al., 1995). Histologically, increasing numbers of chondrocytes will undergo degradation and necrosis and the articular surface will be further damaged showing fibrillation, detachment, and thinning (Dijkgraaf, et al., 1995; de Bont, et al.,

1985b). The collagen network shows further disorganization and loss of metachromasia in the extracellular matrix when staining for proteoglycan content (de Bont, et al., 1985; Hough, et al., 1989; Dijkgraaf, et al., 1995). Biochemically, synthesis of extracellular matrix components has been inhibited and synthesis of proteases has increased due to constant stimulus by cytokines IL-1 and TNF-α (Dijkgraaf, et al., 1995). It has been reported that osteoarthritic cartilage has increased cytokine receptors on the chondrocyte cell membrane making Il-1 and TNF-α more apt to stimulate production of proteases, especially metalloproteinases, and inhibit any attempt by the chondrocytes to repair the damage (Aigner, et al., 2006; Fernandes, et al., 2002; McIlwraith, 1996a; Lefevre, et al., 1990). Matrix metalloproteinases (MMPs) and aggrecanases (ADAMTS) are considered key players in the degradation of the extracellular matrix through their destruction of collagen and proteoglycan (Clegg, et al., 1997; Gerwin, et al., 2006; Tang, et al., 2001; Sandy, et al., 2001; Cal, et al., 2002; Martel-Pelletier, et al., 2000). Tissue inhibitors of metalloproteinases (TIMPs) keep the MMPs and ADAMTS under tight control in normal cartilage, but in osteoarthritic conditions, a deficiency in TIMPs allow the MMPs and ADAMTS to further degrade the extracellular matrix (Woessner, et al., 1991; Yamada, et al., 1987; Dean, et al., 1984; Dean, et al., 1987). In addition to stimulating synthesis and release of other cytokines and proteolytic enzymes, IL-1 also stimulates the chondrocytes, synovial cells and inflammatory cells to synthesize and release arachidonic acid metabolites such as prostaglandin E_2 (PGE_2) and leukotrienes (May, et al., 1981; Dayer, et al., 1986; Todhunter, et al., 1990: von Rechenberg, et al., 2000). PGE_2 in joints have been shown to have several effects ranging from enhancement of pain perception and vasodilation to proteoglycan depletion from the extracellular matrix and bone

demineralization (McIlwraith, 1996a; Attur, et al., 2008; Miyaura, et al., 2000; Stock, et al., 2001).

2.3 Current Treatment of Osteoarthritis

Current therapy for osteoarthritis consists of both medical and surgical options designed to control inflammation as well as minimize further damage to the articular cartilage and mitigate degenerative changes associated with progressing stages of osteoarthritis. Medical therapy consists of rest/physical therapy, oral joint supplements such as glucosamine and chondroitin sulfate, and oral or injectable medications such as nonsteroidal anti-inflammatories, corticosteroids, hyaluronic acid, and polysulfated glycosaminoglycans (Goodrich, et al. 2006). The recent development of interleukin-1 receptor antagonist protein (IRAP) is also another viable therapeutic option (Frisbie, et al., 2002). Surgical options for treatment of osteoarthritis involve joint lavage, arthroscopy/arthrotomy, or arthrodesis (McIlwraith, et al., 1996b). Recently, there have also been attempts at surgical joint resurfacing in an effort to repair cartilage defects that could eventually lead to chronic inflammation and osteoarthritis (McIlwraith, et al., 1996c). With all these therapeutic options available, the mainstay of treatment regimes for allowing horses to return promptly to athletic function remains intra-articular injections of corticosteroids and hyaluronic acid (Frisbie, et al., 1997).

2.3.1 Corticosteroids

Corticosteroids are potent anti-inflammatory agents that are routinely injected into equine joints to minimize pain and inflammation associated with trauma-induced joint

disease (Trotter1996b). Controversy still remains over relative risk/benefit ratios associated with intra-articular corticosteroid use. However, recent studies have focused on the intra-articular effects of corticosteroids on both the biochemical and morphologic changes within the articular cartilage with and without exercise, and the metabolism of the extracellular matrix by the chondrocytes and hyaluronic acid production by the synovial membrane (Trotter 1991; Shoemaker 1992; Foland 1994; Frisbie 1997; Frisbie 1998; Todhunter 1993; Roneus 1993; Tulamo RM, 1991). Ultimately, judicious use, as well as type of corticosteroid used, concentration, duration of exposure, and other tissue and cell variables play a role in the corticosteroid effects on articular cartilage (Goodrich 2006).

All corticosteroids consist of twenty-one carbon molecules that are arranged in three six-carbon rings and one five-carbon ring. The biologically active form contains a β-hydroxyl group at C-11 of the molecule and thus does not require biotransformation in the liver (Trotter 1996b). Solubility of synthetic corticosteroid preparations determines rate of absorption and duration of action. Acetate and acetonide esters, such as methylprednisolone acetate and triamcinolone acetonide, are more lipid soluble and as a result have a delayed absorption rate and a longer duration of action (Trotter 1996b). Another important factor to consider in the duration and onset of action is the rate of hydrolysis of the corticosteroid to its active moiety. Once in the active form, the corticosteroid may exert its effects at the cellular level (Trotter 1996b).

Glucocorticoids have been shown to bind to specific glucocorticoid receptors present on neutrophils, monocytes, lymphocytes, and eosinophils which act to modulate expression of certain genes inhibiting action of inflammatory cytokines (IL-1 and NF-

κB), thus significantly decreasing the inflammatory reaction (Boumpas, et al., 2001; Gilron, 2004). Probably the most recognized effect of corticosteroids is their anti-inflammatory action through inhibition of prostaglandin production. Corticosteroids act through lipocortin-induced phospholipase inhibition preventing the mobilization of phospholipids within the cell membranes to become available for the arachidonic acid cascade, ultimately resulting in inhibition of production of prostaglandins and leukotrienes responsible for pain and inflammation (Di Rosa, 1985; Gilron, 2004).

The commonly used corticosteroids for equine joint injections include methylprednisolone acetate (Depo-Medrol), triamcinolone acetonide (Kenalog), and betamethasone sodium phosphate/betamethasone acetate (Celestone Soluspan). Several studies, both *in vitro* and *in vivo*, have been performed looking at the beneficial and detrimental effects of these corticosteroids on the articular cartilage (Dechant, et al., 2003; Doyle, et al., 2005; Foland, et al., 1994; Frisbie, et al., 1997; Frisbie, et al., 1998; Fubini, et al., 2001; MacLeod, et al., 1998; Murphy, et al., 2000; Roneus, et al., 1993; Sandler, et al., 2004; Shoemaker, et al., 1992; Todhunter, et al., 1993; Todhunter, et al., 1996; Trotter, et al., 1991; Tulamo 1991; Yates, et al., 2006). Of these three corticosteroids, methylprednisolone has the longest duration of action against inflammation associated with osteoarthritis, followed by betamethasone and then triamcinolone (Frisbie, 2006). Detrimental effects of methylprednisolone include decreased proteoglycan synthesis, increased proteoglycan degradation, decreased mRNA expression of collagen type II and aggrecan, and articular cartilage erosion (Doyle, et al., 2005; Murphy, et al., 2000; Todhunter, et al., 1996; Yates, et al., 2006; Frisbie, et al., 1998). However, at lower physiologic doses, methylprednisolone has been shown to

exert some protective effects (Farquhar, et al., 1996; Murphy, et al., 2000; Todhunter, et al., 1996). In contrast, triamcinolone has been demonstrated to have mostly beneficial effects on articular cartilage (Frisbie, et al., 1997; Sandler, et al., 2004; Dechant, et al., 2003). An *in vivo* study showed triamcinolone minimized the development of secondary osteoarthritis in an osteochondral fragment model (Frisbie, et al., 1997). A recent *in vitro* study demonstrated that triamcinolone may inhibit degradative enzymes, such as matrix metalloproteinases, while protecting gene transcription of extracellular matrix factors (Richardson, et al., 2003). Both *in vitro* and *in vivo* work evaluating these corticosteroids would indicate that triamcinolone is best recommended for intra-articular use, especially in high-motion joints (Frisbie, 2006).

2.3.2 Hyaluronic Acid

Hyaluronic acid is a polyanionic nonsulfated glycosaminoglycan consisting of repeating disaccharide units of D-glucoronic acid and N-acetylglucosamine in a long unbranched chain, forming particles of widely varying sizes (Howard, et al., 1993). The molecular weight of hyaluronic acid measured in synovial fluid has been reported in the range of 0.5 to 3.0 million daltons (Tew, 1984; Tulamo, et al., 1994). However, the maximal limit for molecular mass determination using the high-performance liquid chromatography-gel exclusion chromatography technique was 3 million daltons, leaving open the possibility of much higher molecular weight hyaluronic acid molecules within the synovial fluid and extracellular matrix (Tulamo, et al., 1994). Hyaluronate concentrations within the synovial fluid can vary within one individual depending on various joints, with smaller joints generally having a higher concentration (Auer, et al.,

1980). Hyaluronate concentrations in normal equine synovial fluid range from 0.33 to 1.5 mg/mL depending on the technique used to quantify the amount (Howard, et al. 1993). The half-life of exogenous hyaluronic acid injected into normal equine joints has been estimated around 96 hours (Hilbert, et al., 1985). Conversely, half-life of exogenous hyaluronic acid and its clearance from the joint are significantly reduced in diseased joints (Fraser, et al., 1993).

The specific mechanism of action of hyaluronic acid is unknown. However, it has been theorized that exogenous hyaluronate preparations supplement the actions of depleted or depolymerized endogenous hyaluronate in the synovial fluid, and results in restoration of viscoelasticity, steric hindrance and lubrication of the synovium (Howard, et al., 1993). Anti-inflammatory effects of hyaluronate have also been demonstrated. *In vitro* studies have shown reduction of macrophage chemotaxis and phagocytosis and decreased proliferation and migration of lymphocytes (Balazs, et al., 1985; Forrester, et al., 1981; Swanstrom, 1978). Hyaluronate has also been shown to inhibit neutrophil-mediated degradation of articular cartilage through decreased release of sulfated glycosaminoglycans (Tobetto, et al., 1994). An intravenous preparation of hyaluronic acid was evaluated in horses with experimentally induced osteochondral fragments and found to reduce clinical lameness, reduce articular cartilage thinning and fibrillation, and reduce levels of intra-articular prostaglandins when compared with the saline treated group (Kawcak, et al., 1997). However, it remains uncertain if hyaluronic acid acts only by inhibitory effects on inflammation or if there is a secondary chondroprotective mechanism (Howard, et al., 1993).

There is still much debate over the importance of molecular weight in the efficacy of exogenous hyaluronic acid therapy. Aviad et al. postulated that the beneficial effects of hyaluronic acid were due to its pharmacological properties of cellular modulation rather than the physical properties, such as molecular weight (Aviad, et al., 1994). A clinical study evaluated a low molecular weight hyaluronate versus a high molecular weight product in racing Thoroughbreds with carpitis and showed no clinically significant differences between the two treatments (Aviad et al., 1988). A met analysis study concluded that medium molecular weight hyaluronic preparations demonstrated the most beneficial effects by restoring rheological properties of the synovial fluid and reducing indices of synovial inflammation (Ghosh, et al., 2002). Conversely, an *in vitro* study by Smith et al. demonstrated that hyaluronic acid with a molecular weight of greater than $5x10^5$ daltons stimulated the synthesis of hyaluronate in a dose-dependent manner, while hyaluronic acid preparations less than $5x10^5$ daltons had little or no effect except at high concentrations where synthesis was still depressed (Smith, et al., 1987). A comparative study of five different hyaluronic acid preparations in racehorses with traumatic arthritis was performed and demonstrated longer duration of soundness with preparations of molecular weights of $2x10^6$ daltons or greater (Phillips, 1989). More recent studies have further documented the beneficial effects of high molecular weight hyaluronic acid preparations on the articular chondrocytes through inhibition of phagocytosis and migration, restoration of mean molecular weight levels to levels obtained prior to induction of carpitis, and inhibition of PGE2 production. (Forrester, et al., 1980; Akatsuko, et al., 1993; White, et al., 1999; Campo, et al., 2009).

2.3.3 Combination Treatment

The use of hyaluronic acid in combination with corticosteroids has become a popular therapeutic approach by many clinicians. However, scientific evidence to support the combination therapy is minimal. One of the first reports of combination therapy concluded that the grouping of hyaluronic acid with a corticosteroid resulted in better and longer lasting improvement in lameness than corticosteroids alone (Rydell, et al., 1970). Another study suggested that combination of hyaluronic acid with either methylprednisolone or betamethasone mitigated the deleterious effects of biochemical degradation seen with repeated corticosteroid injections (Roneus, et al., 1993). More recent studies looked at the effects of a medium-molecular weight hyaluronic acid in combination with methylprednisolone both on normal cartilage explants and IL-1 induced chondrocyte pellets (Doyle, et al., 2005; Yates, et al., 2006). These studies concluded that there was no beneficial effect of combination therapy on normal cartilage explants, and hyaluronic acid treatment did little to mitigate the proteoglycan catabolism caused by corticosteroid treatment (Doyle, et al., 2005). However, there was a beneficial effect on proteoglycan metabolism in the combination therapy when the treatments were applied to chondrocytes that were exposed to IL-1. (Yates, et al., 2006). The purpose of this study was to evaluate the effects of two of the most commonly used intra-articular therapies in high motion joints. This included a medium-acting corticosteroid (triamcinolone) and a high molecular weight hyaluronic acid (Hylartin-V). Our hypothesis was that administration of hyaluronic acid alone or in combination with triamcinolone would mitigate the chondrocyte proteoglycan catabolism caused by IL-1 administration.

CHAPTER 3. MATERIALS AND METHODS

3.1 Pellet Culture

All horses used in this study were euthanized by a lethal dose of sodium pentobarbital administered intravenously for reasons unrelated to joint disease. The Institutional Animal Care and Use Committee at the University of Illinois approved this study. Articular cartilage was aseptically collected from the fetlock joints of 10 horses, ranging in age from 2 to 4 years. All joints were evaluated to ensure there was no gross evidence of joint disease prior to cartilage collection. The cartilage was placed in chondrogenic media consisting of Dulbecco modified Eagle medium,[a] 10% fetal bovine serum,[b] 1% L-glutamine,[c] 1% penicillin-streptomycin,[d] and ascorbic acid (50 µg/mL),[e] and digested overnight with 0.2% collagenase.[f] After digestion, an estimation of total chondrocyte number and viability was made using a Reichart hematocytometer and Trypan blue stain.[g] The chondrocytes were resuspended at a concentration of 500,000 cells/mL in chondrogenic media. The media containing the chondrocytes was transferred to Eppendorf tubes (0.5mL/tube). The media was centrifuged to form chondrocyte pellets containing 250,000 cells. The pellets were incubated at 37ºC for 7 days to allow formation of an extracellular matrix and the media was changed every 2 to 3 days.

Treatments were administered on day 7 and the pellets were incubated for an additional 24 hours. There were 10 treatment groups with a minimum of 10 pellets in each group. Treatment groups (Table 1) consisted of fresh media only (negative control), fresh media with 10 ng/mL of IL-1[h] only (positive control), IL-1 (10 ng/mL) and HA[i] (0.5 mg/mL or 2 mg/mL; 2 treatment groups), IL-1 (10 ng/mL) and TA[j] (0.06 mg/mL or 0.6 mg/mL; 2 treatment groups), and IL-1 (10 ng/mL) with both HA (0.5 mg/mL or 2

mg/mL) and TA (0.06 mg/mL or 0.6 mg/mL; 4 treatment groups). The concentrations of HA and TA were determined from a range of published concentrations likely to be present in the fetlock joint 24 to 48 hours after an intra-articular injection (Chen, et al., 1992; Hilbert, et al., 1985). At the time of treatment, 4 pellets from each treatment group were radiolabeled with media containing sulfur 35 (S^{35})-labeled sodium sulfate (10 μCi/mL).[k] All pellets were removed from the treatment media after 24 hours of incubation and were washed 3 times with PBS solution. Seven horses had 4 radiolabeled pellets and exhausted media stored at -80ºC until further analysis. Three horses had 20 pellets in each treatment group snap frozen in liquid nitrogen and saved at -80ºC for RNA isolation. Six horses had 2 pellets per treatment group saved for histological examination.

3.2 Proteoglycan Synthesis

New proteoglycan synthesis was determined by $^{35}SO_4$ incorporation into the pellet content and subsequent release into the media over a 24 hour period. Radiolabeled pellets were digested in papain (150 μg/mL)[l] at 65ºC for 24 hours. Radiolabeled media was digested in papain (150 μg/mL) at 65ºC for 4 hours. Aliquots of 25 μL radiolabeled papain-digested pellets and 25 μL radiolabeled papain-digested media were placed in 96-well MultiScreen filtration plates,[m] precipitated with 0.2% Alcian blue dye solution, and counted for scintillation (Masuda, et al., 1994). Radio-isotope decay was accounted for in all values.

3.3 Total Pellet GAG Content

Total GAG content in the pellets and in the media was determined using a dimethylmethylene blue binding assay (Farndale, et al., 1982). Pellets were digested in

papain as described previously. Aliquots of 25 μL papain-digested pellets were placed into 96-well microplates, 200 μL of 1,9-Dimethylmethylene Blue dye[n] was added, and samples were analyzed for absorbance. All samples were compared against a standard curve of chondroitin sulfate to estimate the total GAG content of the digested pellets.

3.4 Total Pellet DNA Content

Total DNA content of each pellet was determined using a fluorometric Hoechst assay (Kim, et al., 1988). Pellets were digested in papain as described previously. Aliquots of 10 μL papain-digested pellets were placed into 96-well microplates, 200 μL of Hoechst dye solution[o] was added, and samples were analyzed for fluorescence in a microplate reader.[p] All samples were compared against a standard curve of calf thymus to estimate total DNA content of the digested pellets.

3.5 Pellet mRNA Levels

Real-time PCR data was obtained from chondrocyte pellet cultures of 3 horses. The RNA was extracted from 20 pellets in each treatment group per horse using the Trizol reagent[q] according to the manufacturer's suggested protocol. Complementary DNA was obtained by priming the sample with oligo d(T)[r] and then adding reverse transcriptase.[s] Real-time quantitative PCR analysis was performed for collagen type II, aggrecan, and COX-2, and normalized to elongation factor-1α mRNA expression. A PCR detection system[t] was used to perform the assay (Doyle, et al., 2005; Yates, et al., 2006; Livak and Schmittgen, 2001).

3.6 Histologic Examination

After 24 hours in 4% paraformaldehyde, pellets were transferred to a 4% agarose gel and stored at 4ºC overnight. The pellets were dehydrated in alcohol, embedded in paraffin, sectioned, and stained with Toluidine blue.

3.7 Statistical Analysis

All non-normally distributed data were logarithmically transformed and presented as mean ± SE log values. Significance of IL-1 was determined by using a one-way repeated-measures ANOVA generated through the SigmaStat[u] software program comparing the positive control (IL-1) to the negative control (no IL-1). Significance of HA and TA was determined using a two-way repeated-measures ANOVA generated through the SigmaStat[u] software program. All post hoc tests were conducted when indicated by use of the Holm-Sidak method. Values of $P \leq 0.05$ were considered significant.

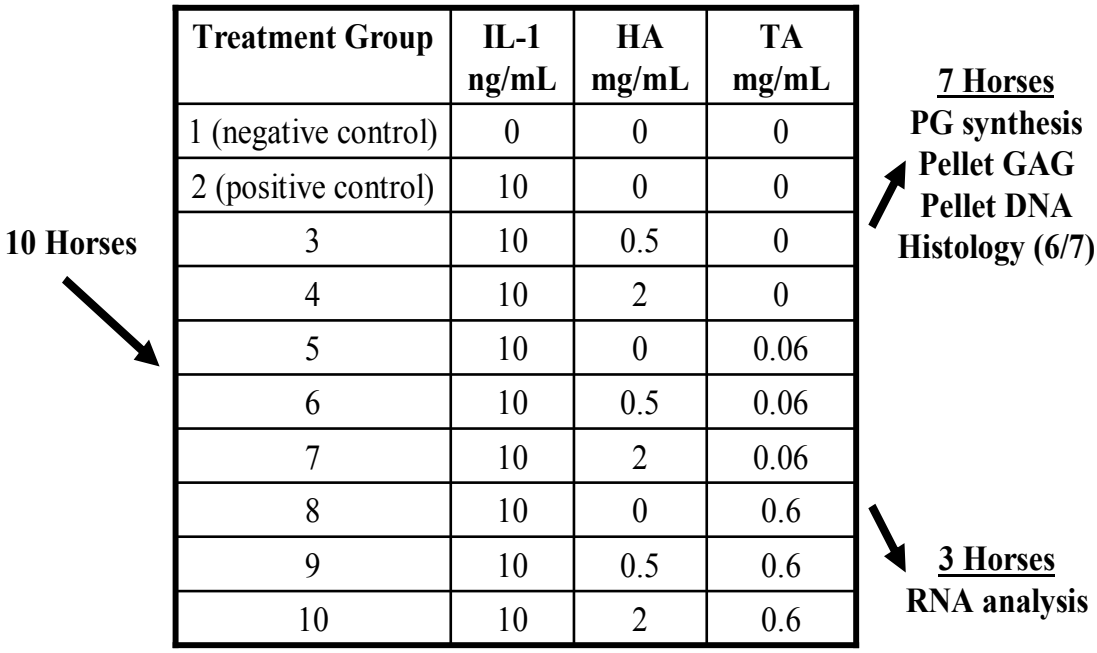

Table 1. Treatment Groups Chondrocytes were collected from 10 horses. Each horse was run as a separate experiment. All experiments had the same 10 treatment groups. Seven horses were collected for PG synthesis, pellet GAG content, and pellet DNA content. Only 6 of these 7 horses were evaluated histologically. Three horses were collected for RNA analysis.

CHAPTER 4. RESULTS

4.1 Pellet GAG Synthesis

Pellet GAG synthesis was designated as the amount of newly synthesized GAG retained in the pellet (CPM per chondrocyte pellet). Treatment with IL-1 significantly ($P = 0.018$) decreased GAG synthesis in the positive control (IL-1 only) when compared to the negative control (no IL-1; Figure 1). Treatment with HA (2 mg/mL) significantly ($P < 0.001$) increased GAG synthesis when compared to the IL-1 control group. The 0.006 and 0.6 mg/mL TA treatment groups did not have a significant ($P = 0.218$) effect on GAG synthesis when compared to the 0 mg/mL TA treatment group. However, there was a significant ($P = 0.004$) interaction with the combined treatment of HA and TA on increasing GAG synthesis. Specifically, 2 mg/mL of HA combined with 0.06 or 0.6 mg/mL of TA significantly increased GAG synthesis compared to the IL-1 control group.

4.2 Total GAG Synthesis

Total GAG synthesis was designated as the amount of newly synthesized GAG retained in the pellet and released into the media (CPM pre chondrocyte pellet + CPM per media). Treatment with IL-1 showed no significant ($P = 0.097$) difference in total GAG synthesis when the positive control (IL-1 only) was compared to the negative control (no IL-1; Figure 2). Treatment with HA (2 mg/mL) showed a significant ($P = 0.026$) increase in total GAG synthesis when compared to the IL-1 control group. Treatment with TA did not show a significant ($P = 0.607$) effect on total GAG synthesis. There was no significant ($P = 0.48$) interaction with HA and TA combined on total GAG synthesis.

4.3 Percent GAG Retained in the Pellet and Released into the Media

Percent GAG retained in the pellet was designated as the percentage of newly synthesized GAG retained in the pellet (CPM per pellet/[CPM per pellet + CPM per media]). Percent GAG released into the media was designated as the percentage of newly synthesized GAG released into the media (CPM pre media/[CPM per pellet + CPM per media]). Treatment with IL-1 significantly ($P = 0.04$) decreased the percent GAG retained within the pellets and significantly ($P = 0.04$) increased the percent GAG released into the media when the positive control (IL-1 only) was compared to the negative control (no IL-1; Figure 3, Figure 4). Treatment with HA did not have a significant ($P = 0.26$) effect on percent GAG retained within the pellet or on the percent GAG released into the media ($P = 0.26$) when compared to the IL-1 control group. Treatment with TA showed a significant ($P = 0.004$) increase in percent retention of newly synthesized GAG within the pellet and a significant ($P = 0.004$) decrease in percent newly synthesized GAG released into the media. Specifically, the 0.06 and 0.6 mg/mL TA groups had a significant increase in percent GAG retained within the pellets and a significant decrease in percent GAG released into the media when compared to the 0 mg/mL TA treatment group. There was no significant ($P = 0.67$) interaction with HA and TA combined on percent GAG retained in the pellet of percent GAG released into the media.

4.4 Total GAG Pellet Content

Total pellet GAG content was designated as the total GAG content retained in the pellet after treatment and this included the newly synthesized GAG (μg per chondrocyte pellet). Treatment with IL-1 did not have a significant ($P = 0.101$) effect on the total

pellet GAG content when the positive control (IL-1 only) was compared to the negative control (no IL-1; Figure 5). Treatment with HA (2 mg/mL) significantly ($P = 0.002$) increased the total GAG content within the pellet when compared to the IL-1 control group. Treatment with TA (0.6mg/mL) significantly ($P = 0.036$) increased total GAG pellet content when compared to the 0 mg/mL TA treatment group. There was no significant ($P = 0.732$) interaction with HA and TA combined on the total GAG pellet content.

4.5 Total DNA pellet content

Total DNA pellet content was designated as the total amount of DNA per pellet (μg per pellet). Treatment with IL-1 ($P = 0.217$), HA ($P= 0.781$), or TA ($P = 0.982$) had no significant effect on the total DNA content per pellet.

4.6 Pellet mRNA Levels

Treatment with IL-1 significantly ($P = 0.026$) increased collagen type II mRNA levels when the positive control (IL-1) was compared to the negative control (no IL-1; Figure 6). Treatment with HA had no significant ($P = 0.102$) effect on collagen type II mRNA levels when compared to the IL-1 control group. Treatment with 0.06 and 0.6 mg/mL TA significantly ($P = 0.001$) decreased collagen type II mRNA levels when compared to the IL-1 control group. There was no significant ($P = 0.121$) interaction of HA and TA combined on collagen type II mRNA levels.

Treatment with IL-1 significantly ($P = 0.045$) increased aggrecan mRNA levels when the positive control (IL-1) was compared to the negative control (no IL-1; Figure 7). Treatment with HA did not have a significant ($P = 0.725$) effect on aggrecan mRNA levels when compared to the IL-1 control group. Treatment with TA had a significant (P

= 0.045) effect upon aggrecan mRNA levels. Specifically, the 0.06 and 0.6 mg/mL TA treatment groups significantly decreased aggrecan mRNA levels when compared to the IL-1 control group. There was no significant (P = 0.11) interaction of HA and TA combined on aggrecan mRNA levels.

Treatment with IL-1 significantly ($P = 0.021$) increased COX-1 mRNA levels when the positive control group (IL-1) was compared to the negative control (no IL-1; Figure 8). Treatment with HA did not have a significant ($P = 0.126$) effect on COX-2 mRNA levels when compared to the IL-1 control group. Treatment with TA had a significant ($P = 0.007$) effect on COX-2 mRNA levels. Specifically, the 0.06 and 0.6 mg/mL TA treatment groups significantly decreased COX-2 mRNA levels when compared to the IL-1 control group. There was no significant ($P = 0.464$) interaction of HA and TA combined on COX-2 mRNA levels.

4.7 Histologic Examination

The chondrocyte pellets varied in size dependent on the horse. The size of the pellets was not measured; instead the pellets were only evaluated for proteoglycan production through use of the toluidine blue stain. Subjective pellets treated with HA, TA, or the combination of HA and TA, had increased proteoglycan staining throughout the pellet matrix (Figure 9).

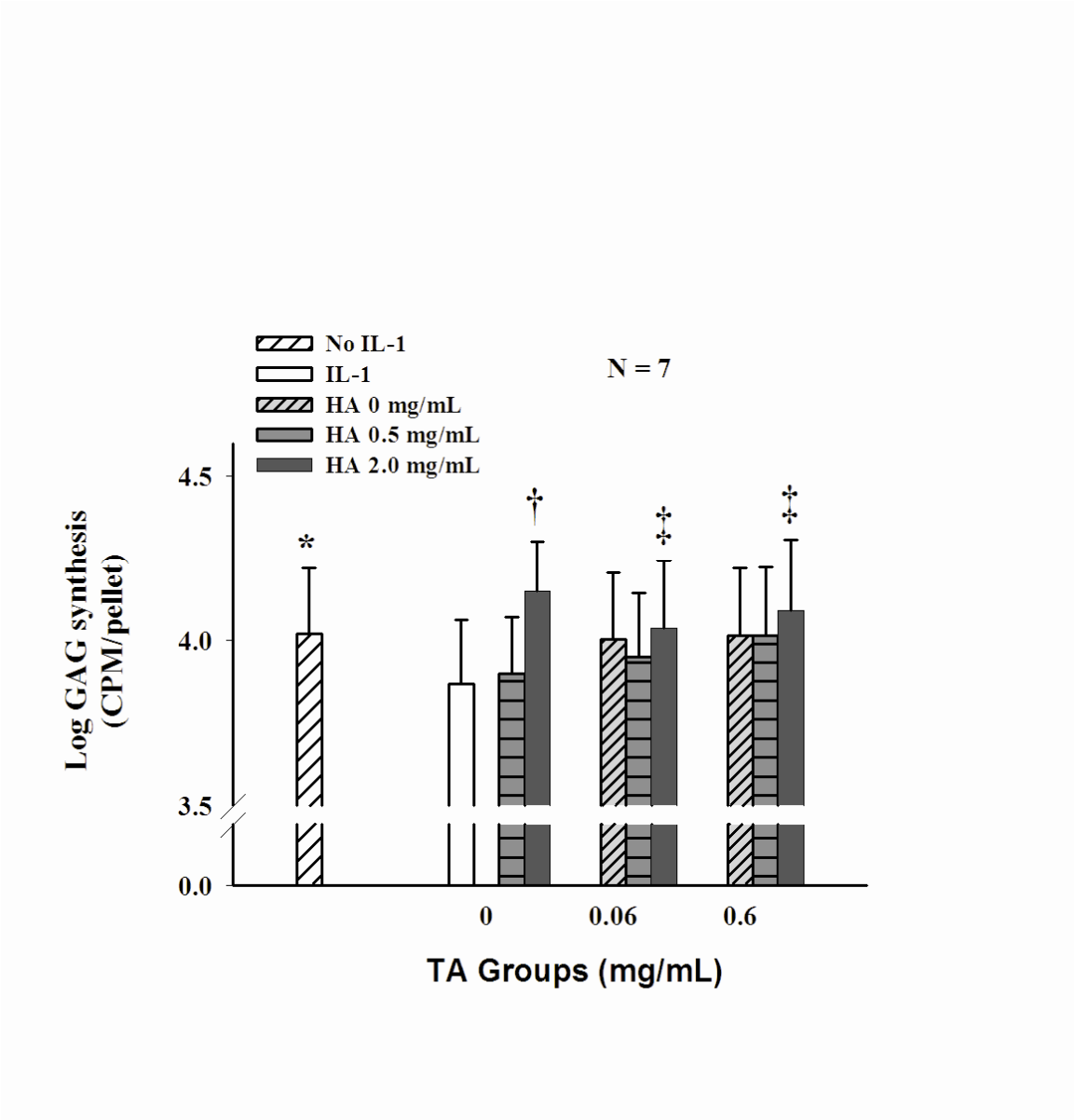

Figure 1. Pellet GAG Synthesis – Log mean ± SE incorporation of sulfur 35-labeled sodium sulfate into GAG of equine articular chondrocyte pellets treated with various concentrations of HA and TA. Scintillation counts (counts per minute [CPM]) were normalized for decay and pellet digestion volume. *Significant ($P < 0.05$) difference between negative control (no IL-1) and positive control (IL-1 only). †Significant ($P < 0.05$) effect of HA compared with no HA at the same concentrations of TA. ‡Significant ($P < 0.05$) effect of combination therapy (HA and TA) compared to the IL-1 control.

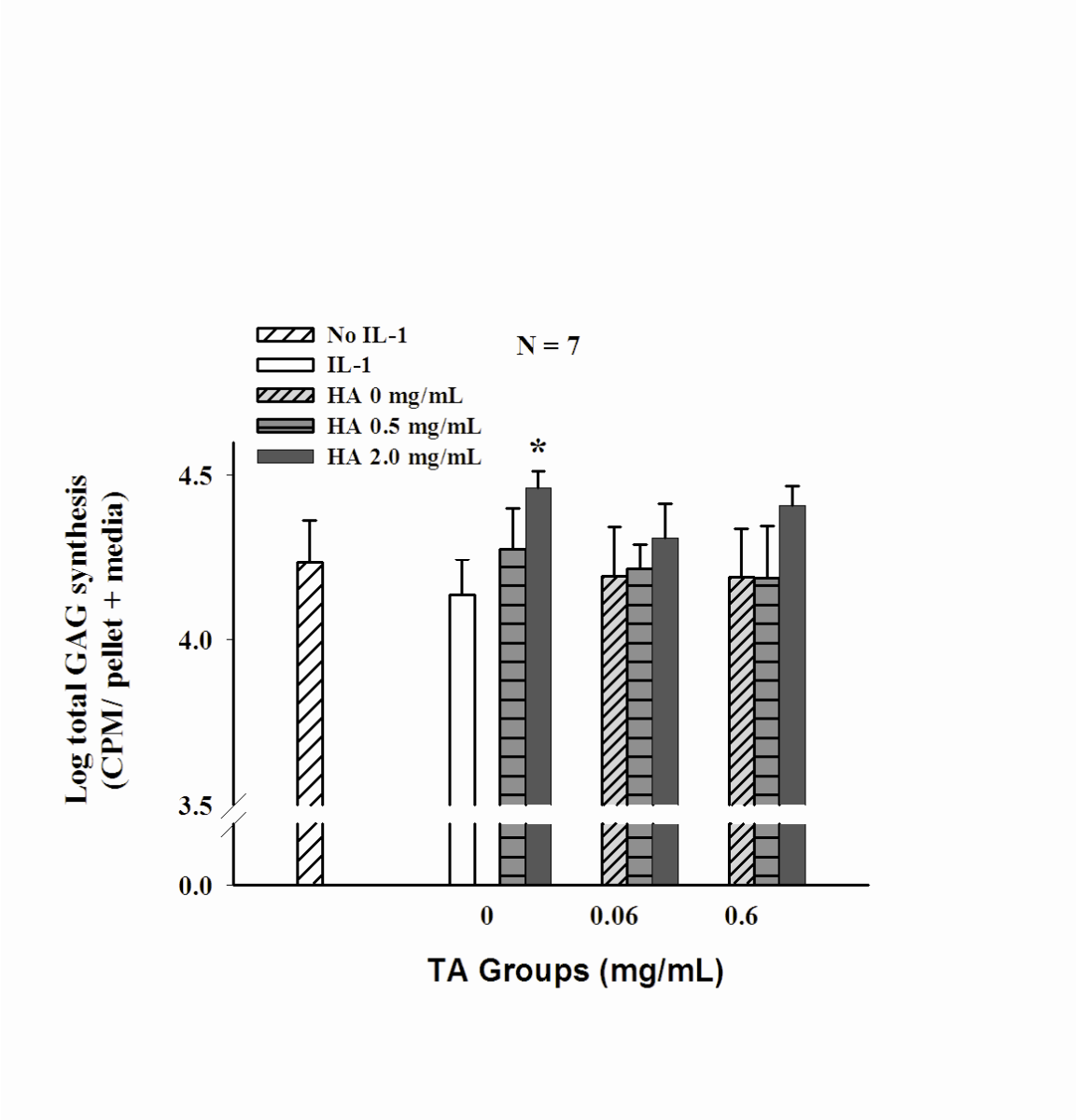

Figure 2. Total GAG Synthesis - Log mean ± SE incorporation of sulfur 35-labeled sodium sulfate into GAG of both equine articular chondrocyte pellets and the media following treatment with various concentrations of HA and TA. Scintillation counts (counts per minute [CPM]) were normalized for decay and pellet digestion volume. *Significant ($P < 0.05$) effect of HA compared with no HA at the same concentrations of TA.

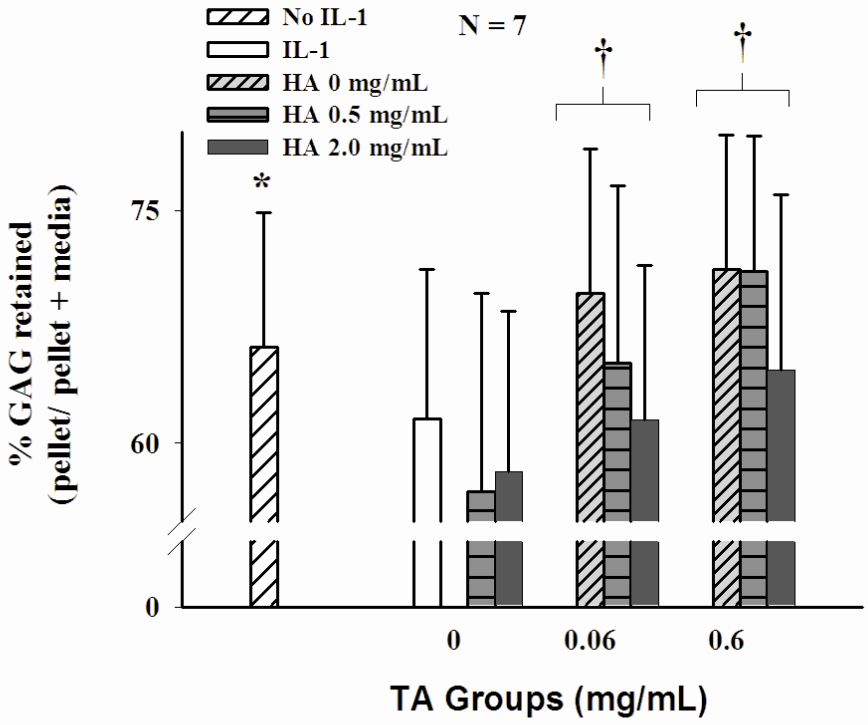

Figure 3. Percent GAG Retained in the Pellet - Percent mean ± SE incorporation of sulfur 35-labeled sodium sulfate into GAG of equine articular chondrocyte pellets treated with various concentrations of HA and TA. Scintillation counts (counts per minute [CPM]) were normalized for decay and pellet digestion volume. *Significant ($P < 0.05$) difference between negative control (no IL-1) and positive control (IL-1 only). †Significant ($P < 0.05$) effect of TA treatment groups compared with the no TA treatment group at all concentrations of HA.

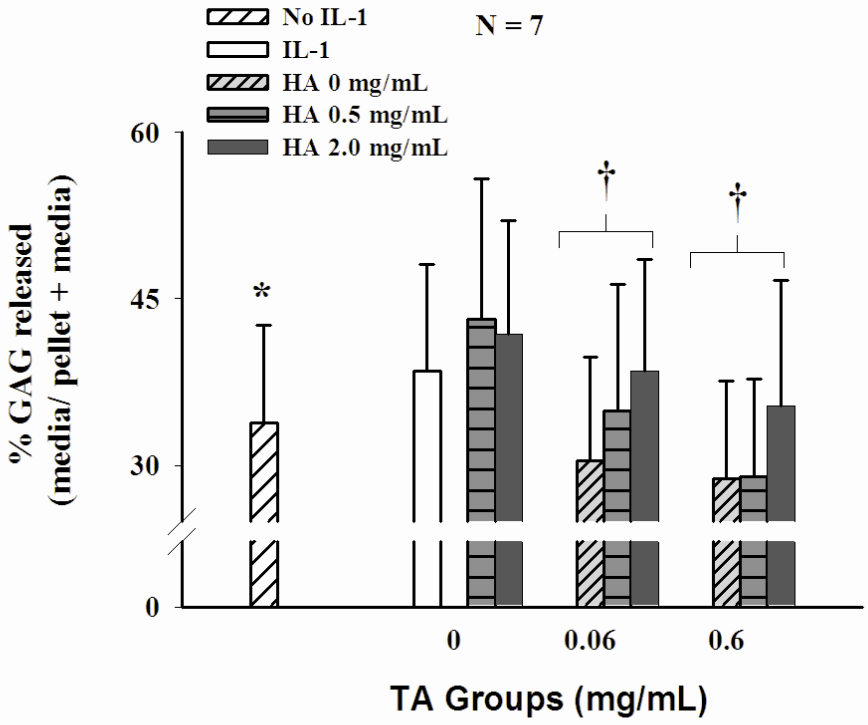

Figure 4. Percent GAG Released into the Media - Percent mean ± SE incorporation of sulfur 35-labeled sodium sulfate into GAG released into media following treatment with various concentrations of HA and TA. Scintillation counts (counts per minute [CPM]) were normalized for decay and pellet digestion volume. *Significant ($P < 0.05$) difference between negative control (no IL-1) and positive control (IL-1 only). †Significant ($P < 0.05$) effect of TA treatment groups compared with the no TA treatment group at all concentrations of HA.

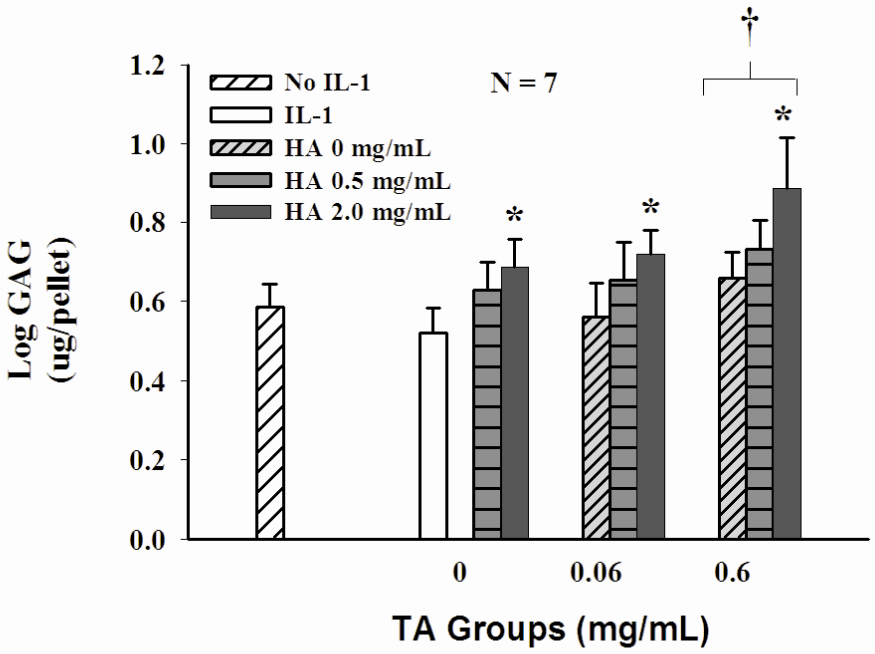

Figure 5. Total GAG Pellet Content - Log mean ± SE total GAG content in equine articular chondrocyte pellets treated with various concentrations of HA and TA. Content of GAG was normalized for pellet digestion volume. *Significant ($P < 0.05$) effect of HA compared with no HA at the same concentrations of TA. †Significant ($P < 0.05$) effect of TA treatment groups compared with the no TA treatment group at all concentrations of HA.

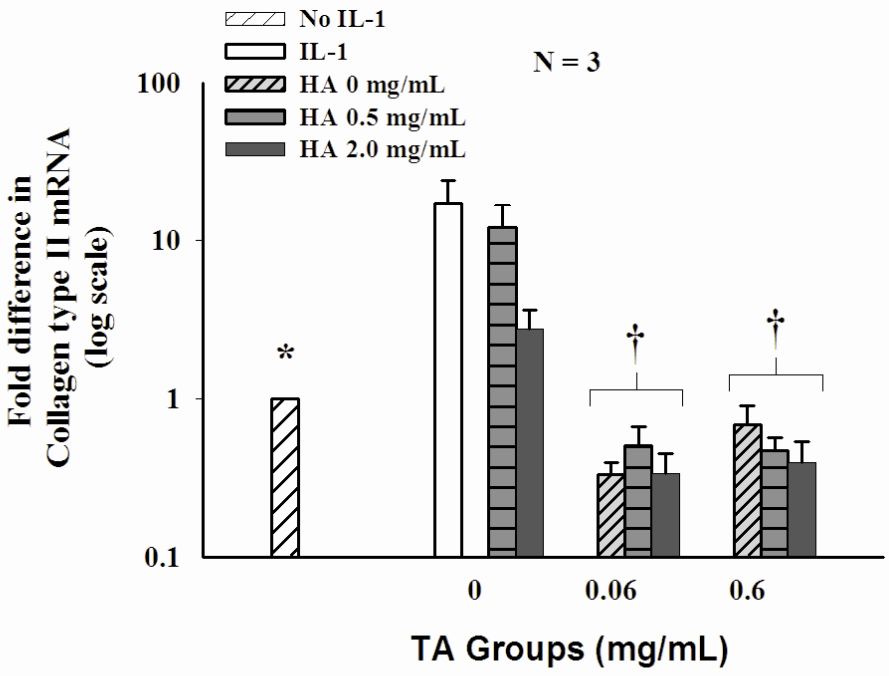

Figure 6. Collagen Type II mRNA Levels - Mean ± SE collagen type II mRNA levels in equine articular chondrocyte pellets treated with various concentrations of HA and TA. Collagen type II mRNA levels were normalized to elongation factor 1-α mRNA levels. *Significant ($P < 0.05$) difference between negative control (no IL-1) and positive control (IL-1 only). †Significant ($P < 0.05$) effect of TA treatment groups compared with the no TA treatment group at all concentrations of HA.

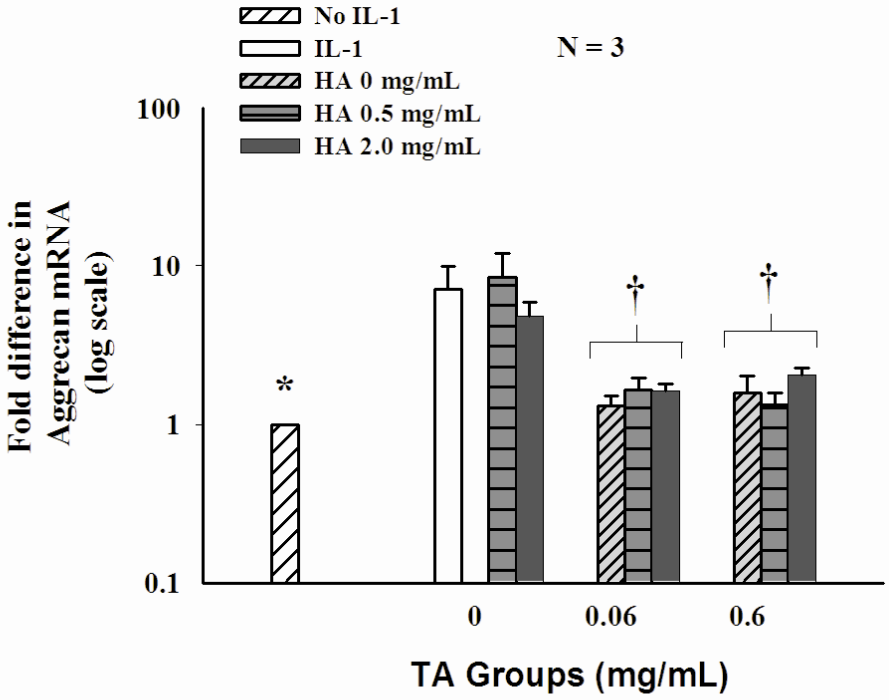

Figure 7. Aggrecan mRNA Levels - Mean ± SE aggrecan mRNA levels in equine articular chondrocyte pellets treated with various concentrations of HA and TA. Aggrecan mRNA levels were normalized to elongation factor 1-α mRNA levels. *Significant ($P < 0.05$) difference between negative control (no IL-1) and positive control (IL-1 only). †Significant ($P < 0.05$) effect of TA treatment groups compared with the no TA treatment group at all concentrations of HA.

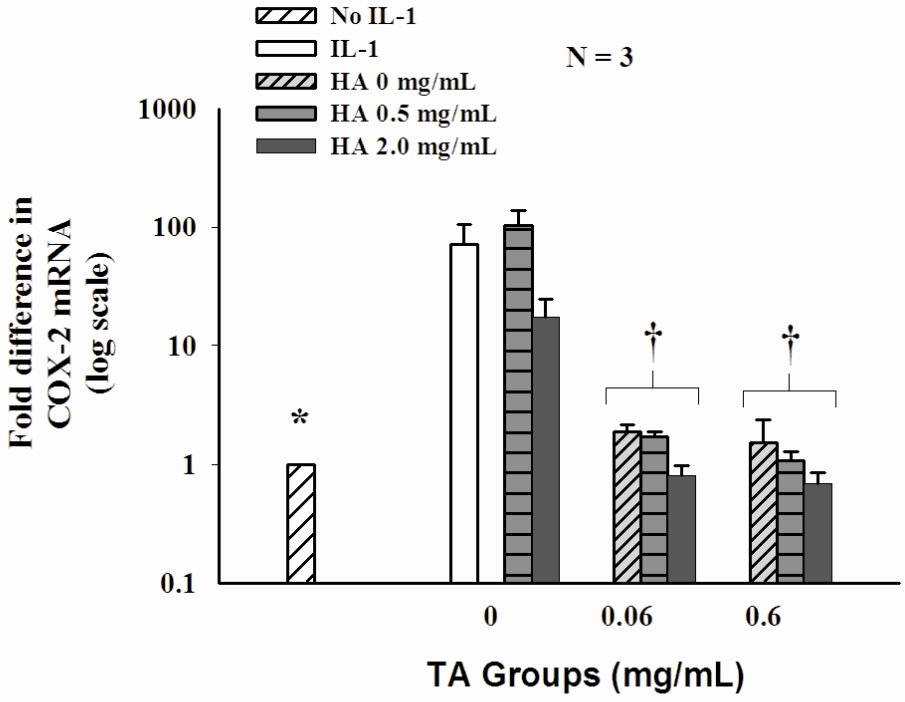

Figure 8. COX-2 mRNA Levels - Mean ± SE COX-2 mRNA levels in equine articular chondrocyte pellets treated with various concentrations of HA and TA. COX-2 mRNA levels were normalized to elongation factor 1-α mRNA levels. *Significant ($P < 0.05$) difference between negative control (no IL-1) and positive control (IL-1 only). †Significant ($P < 0.05$) effect of TA treatment groups compared with the no TA treatment group at all concentrations of HA.

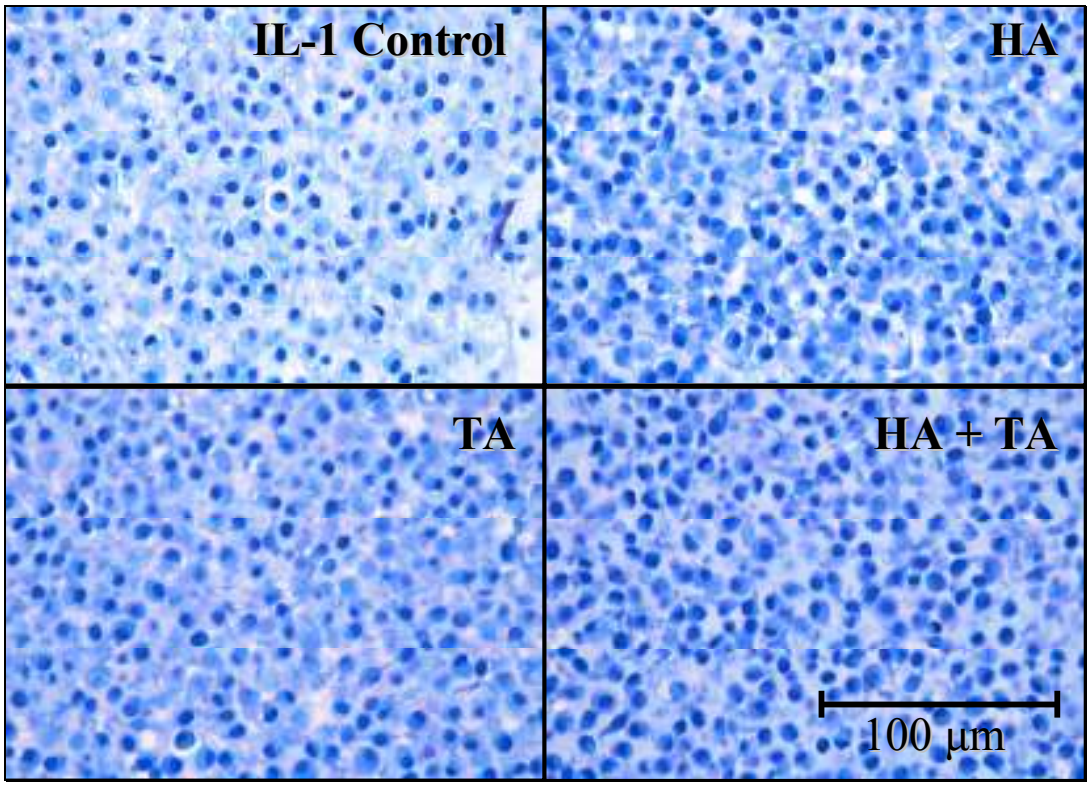

Figure 9. Histologic Examination - Photomicrographs at 40X magnification of sections of equine articular chondrocyte pellets obtained 24 hours after administration of IL-1 treatment, 2 mg/mL HA, 0.6 mg/mL TA, or 2 mg/mL HA and 0.6 mg/mL TA. Toluidine blue stain; bar = 100 µm.

CHAPTER 5. DISCUSSION

Results of this study demonstrated the beneficial effects of HA alone and in combination with TA on IL-1 treated equine articular chondrocyte pellets. HA increased both the new GAG synthesis by the chondrocytes and the total GAG content retained in the pellets when compared to the IL-1 control. Treatment with TA increased the total GAG content retained in the chondrocyte pellet. These results are similar to another study where TA combated the IL-1 induced GAG degradation (Sandler, et al., 2004). Treatment with TA also reduced COX-2 mRNA expression when compared to the IL-1 control. Most of these benefits were seen at the high concentration of HA (2 mg/mL) and the high concentration of TA (0.6 mg/mL). The combination of 2 mg/mL HA and 0.6 mg/mL TA had the highest levels of total pellet GAG following treatment with IL-1.

Treatment with IL-1 had several detrimental effects on chondrocyte metabolism. Administration of IL-1 caused a decrease in GAG synthesis and retention by the pellet ultimately resulting in a decreased newly synthesized pellet GAG content. Treatment with IL-1 also increased COX-2 mRNA expression. In addition, IL-1 administration caused an increase in aggrecan and collagen type II mRNA production. This may be due to an increase in chondrocyte metabolism of aggrecan and collagen type II in response to an IL-1 mediated matrix catabolism (MacLeod, et al., 1998).

Treatment with HA increased GAG synthesis and total GAG pellet content but did not have an effect on mRNA levels of COX-2, aggrecan, or collagen type II. Specifically, addition of HA (2 mg/mL) was beneficial in negating the effects of IL-1 administration and increasing new GAG synthesis. Lower concentrations of HA did not have a significant effect on GAG synthesis. The higher concentrations of HA increased

incorporation of GAG side chains into the pellet matrix. Although HA was beneficial for GAG synthesis, treatment with HA had no effect on COX-2 mRNA levels suggesting that matrix degradation continued to occur in these treatment groups. HA also had no effect on collagen type II and aggrecan mRNA levels, but maintained expression rates similar to the IL-1 treated control group. The disparity between the aggrecan mRNA expression data and the new GAG synthesis suggests that HA may have increased synthesis of products other than aggrecan, such as biglycan and decorin. Alternatively, the retention of aggrecan in the pellet after synthesis may have been more effective in the presence of HA. Ultimately, the proteoglycan synthesis, proteoglycan release, and the total GAG retained in the pellet matrix are probably the most important pieces of information obtained from this study.

Treatment with TA increased total pellet GAG content and decreased mRNA levels of COX-2, aggrecan, and collagen type II. Specifically, treatment with TA increased total GAG pellet content by increasing GAG retention within the pellet while decreasing GAG lost into the media. In addition, TA was beneficial in suppressing COX-2 mRNA levels that increased with IL-1 treatment. As seen in other studies using a corticosteroid, treatment with TA was effective at a cellular level in blocking mediators of inflammation (Yates, et al., 2006; Frisbie, et al., 1998; and Murphy, et al., 2000). In this study, treatment with TA suppressed mRNA levels of aggrecan and collagen type II. However, the TA-treated expression levels were similar to the baseline levels expressed by treatment groups with no IL-1. Corticosteroid-induced suppression of aggrecan and collagen type II mRNA levels has been demonstrated in previous studies (Fubini, et al., 2001). Corticosteroid-induced suppression of aggrecan and collagen type II mRNA levels

may have detrimental effects in a longer term study. These results suggest TA predominantly works by suppressing inflammation to retain GAG in the pellet, not by increasing the synthesis of GAG in the pellet.

Finally, combination treatment of HA and TA mitigated the effects of IL-1 administration by increasing new GAG synthesis and retention within the pellet. The high concentrations of both drugs, HA at 2 mg/mL and TA at 0.6 mg/mL, in combination had the most profound effect on GAG synthesis. These results suggest that HA and TA mitigate the detrimental effects of IL-1 administration through different mechanisms to increase the total GAG content within the pellet. In an inflammatory environment such as that created by IL-1 administration, high concentrations of HA may be beneficial in supporting chondrocyte GAG production while high concentrations of TA may act in an additive or synergistic fashion by retaining GAG within the chondrocyte matrix.

Similar to a previous study, IL-1 was administered to create an inflammatory environment (Yates, et al., 2006). While IL-1 produced expected detrimental effects in this study, other inflammatory mediators are also known to be present in osteoarthritic joints. These pro-inflammatory mediators include tumor necrosis factor-α, interleukin-6, interleukin-8, interleukin-11, interleukin-17, and leukemia inhibitory factor (Shi, et al., 2004). Several studies have documented the detrimental effects of these inflammatory cytokines, suggesting that they may act along different pathways or in a synergistic fashion with IL-1 (Shi, et al., 2004; Schuerwegh, et al., 2003). Results of this study may have been amplified if other pro-inflammatory mediators had been utilized with IL-1 administration to create a model of inflammation.

This study utilized an *in vitro* model to study the effects of inflammation on cartilage metabolism. While an *in vitro* study provided a controlled environment to evaluate the effect of multiple different concentrations of HA and TA on a standardized cartilage matrix, many important *in vivo* influences are lost. Specifically, an *in vitro* study does not allow for clearance of metabolites from the local environment or for systemic metabolism of administered medications. Therefore, these results may not accurately represent what would occur in an *in vivo* model. Further *in vitro* studies evaluating the effects of HA and TA in equine osteoarthritic joints may provide further insight into the benefits and deficiencies of intra-articular HA and TA administration.

The range of HA and TA concentrations used in this study were based on estimated joint concentrations following commonly used intra-articular dosages. In a previous study, TA at 1.2 mg/mL decreased IL-1 induced GAG degradation, but was unable to maintain GAG synthesis and showed an overall decrease in GAG synthesis (Sandler, et al., 2004). In this study, the highest dose of TA (0.6 mg/mL) had a protective effect against IL-1 induced GAG degradation without any decline in GAG synthesis. Based on these findings, it is possible that TA concentrations above 0.6 mg/mL may have some detrimental effects on chondrocytes such as those exhibited with high doses of MPA (Doyle, et al., 2005; Yates et al., 2006; Sandler et al., 2004; and Dechant et al., 2003).

The effects of an intermediate acting corticosteroid in this study were similar to results from a previous study using a similar model and a long acting corticosteroid (Yates, et al., 2006). The response to corticosteroid administration was similar for pellet GAG synthesis, total pellet GAG content, total pellet DNA content, and mRNA

expression. Both MPA and TA increased pellet GAG synthesis in combination with HA suggesting a significant benefit when both corticosteroids and HA are used. Both corticosteroids, MPA and TA, significantly increased the total pellet GAG content. Both MPA and TA had no significant effect on the total pellet DNA content. Finally, MPA and TA both had a significant effect on reducing mRNA levels of COX-2 in the presence of IL-1 administration. The findings of these two studies illustrate the beneficial effects corticosteroids have on reducing inflammation and retaining GAG content in the matrix. These studies also showed the combination of corticosteroids and HA had the most beneficial effect on increasing GAG synthesis of the pellet and retaining the total GAG within the pellet.

In this study, a high molecular weight HA (3,000,000) was used. In previous studies, a medium molecular weight HA (500,000 to 730,000) showed no significant effects on new GAG synthesis when used alone (Doyle, et al., 2005; Yates, et al., 2006). In contrast, this study showed an increase in new GAG synthesis and an increase in total pellet GAG content with only HA administration at 2 mg/mL. These results suggest that a high molecular weight HA may be more beneficial in mitigating IL-1 induced proteoglycan catabolism. However, the efficacy of different molecular weights of HA still remains a controversial subject that needs further conclusive *in vivo* evaluation (White, et al., 1999; Phillips, 1980; and Gosh and Guidolin, 2002).

The highest concentration of HA administered in this study, in combination with TA co-administration, had the most beneficial effects on mitigating IL-1's catabolic actions on proteoglycan matrix metabolism. This effect was a result of both increased GAG synthesis and, more importantly, an increase in retention of pellet GAG through a

decrease in inflammatory mediators. Future studies may be useful to identify the specific inflammatory mediators and matrix-degradative enzymes responsive to IL-1 stimulation and to evaluate the *in vivo* effects of TA and a high molecular weight HA in osteoarthritic equine joints.

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FOOTNOTES

a. DMEM, Mediatech Inc, Herndon, Va.

b. Fetal bovine serum, Gemini Bioproducts, Woodland, Calif.

c. L-glutamine 200mM, Invitrogen, Carlsbad, Calif.

d. Penicillin-streptomycin, BioWhittaker, Cambrex Bio Science, Walkersville, Md.

e. Ascorbic acid, WAKO, Richmond, Va.

f. Collagenase Type 2, Worthington Biomedical Corp, Lakewood, NJ.

g. Trypan Blue stain, 0.4%, Invitrogen, Carlsbad, Calif.

h. Interleukin-1, R&D Systems, Minneapolis, Minn.

i. Hylartin-V, Pfizer Inc, New York, NY.

j. Vetalog, Fort Dodge Laboratories, Fort Dodge, Iowa.

k. S-35-labeled sodium sulfate, MP Biochemicals, Irvine, Calif.

l. Papain, Sigma-Aldrich, St Louis, Mo.

m. Multiwell punch plates, PDVF plate, Millipore, Bedford, Mass.

n. 1,9-Dimethyl-Methylene Blue, Sigma-Aldrich, St Louis, Mo.

o. Hoechst 33258, Sigma-Aldrich, St Louis, Mo.

p. Microplate reader, FLUOstar Optima, BMG Laboratories, Durham, NC.

q. Trizol, Invitrogen, Carlsbad, Calif.

r. Oligo d(T), Invitrogen, Carlsbad, Calif.

s. Superscript II, Invitrogen, Carlsbad, Calif.

t. iCycler iQ real-time PCR detection system, BioRad Laboratories, Hercules, Calif.

u. SigmaStat, version 3.0, Systat Software Inc, San Jose, Calif.

APPENDIX A. ALCIAN BLUE PELLET DATA

Horse	Tx Group	IL-1 ug/mL	HA mg/mL	TA mg/mL	Raw Data	Avg for replicate	Conv Decay	Total Pellet	Avg for tx group
1	1	0	0	0	14935	14877	15119	60478	37439
1	1	0	0	0	14820				
1	1	0	0	0	20974	22603	22971	91884	
1	1	0	0	0	24232				
1	1	0	0	0	16481	15717	15973	63893	
1	1	0	0	0	14953				
1	1	0	0	0	22211	20481	20814	83257	
1	1	0	0	0	18751				
1	2	10	0	0	13470	13789	14013	56055	29891
1	2	10	0	0	14108				
1	2	10	0	0	16388	17137	17415	69663	
1	2	10	0	0	17885				
1	2	10	0	0	14869	13745	13969	55876	
1	2	10	0	0	12621				
1	2	10	0	0	15024	14154	14384	57536	
1	2	10	0	0	13284				
1	3	10	0.5	0					
1	3	10	0.5	0					
1	3	10	0.5	0					
1	3	10	0.5	0					
1	3	10	0.5	0					
1	3	10	0.5	0					
1	3	10	0.5	0					
1	3	10	0.5	0					
1	4	10	2.0	0	10755	10588	10760	43043	25304
1	4	10	2.0	0	10421				
1	4	10	2.0	0	15814	16308	16573	66295	
1	4	10	2.0	0	16802				
1	4	10	2.0	0	11020	10640	10813	43254	
1	4	10	2.0	0	10261				
1	4	10	2.0	0	11580	12262	12461	49845	
1	4	10	2.0	0	12943				
1	5	10	0	0.06	21226	21520	21870	87480	53406
1	5	10	0	0.06	21813				
1	5	10	0	0.06	23596	23757	24144	96577	
1	5	10	0	0.06	23919				
1	5	10	0	0.06	21272	21366	21713	86855	
1	5	10	0	0.06	21460				
1	5	10	0	0.06	39448	38460	39085	156342	
1	5	10	0	0.06	37472				
1	6	10	0.5	0.06					
1	6	10	0.5	0.06					

1	6	10	0.5	0.06					
1	6	10	0.5	0.06					
1	6	10	0.5	0.06					
1	6	10	0.5	0.06					
1	6	10	0.5	0.06					
1	6	10	0.5	0.06					
1	7	10	2.0	0.06	17575	18531	18833	75332	55675
1	7	10	2.0	0.06	19487				
1	7	10	2.0	0.06	26082	27381	27826	111307	
1	7	10	2.0	0.06	28680				
1	7	10	2.0	0.06		30937	31440	125762	
1	7	10	2.0	0.06	30937				
1	7	10	2.0	0.06	32959	32719	33251	133006	
1	7	10	2.0	0.06	32479				
1	8	10	0	0.6	18935	19023	19333	77333	42324
1	8	10	0	0.6	19112				
1	8	10	0	0.6	20253	19877	20200	80801	
1	8	10	0	0.6	19501				
1	8	10	0	0.6	21875	23540	23923	95694	
1	8	10	0	0.6	25206				
1	8	10	0	0.6	21597	20852	21191	84767	
1	8	10	0	0.6	20108				
1	9	10	0.5	0.6					
1	9	10	0.5	0.6					
1	9	10	0.5	0.6					
1	9	10	0.5	0.6					
1	9	10	0.5	0.6					
1	9	10	0.5	0.6					
1	9	10	0.5	0.6					
1	9	10	0.5	0.6					
1	10	10	2.0	0.6	8934	9556	9711	38847	25024
1	10	10	2.0	0.6	10178				
1	10	10	2.0	0.6	7293	7009	7123	28493	
1	10	10	2.0	0.6	6725				
1	10	10	2.0	0.6	17812	17784	18073	72293	
1	10	10	2.0	0.6	17756				
1	10	10	2.0	0.6	17130	14897	15139	60558	
1	10	10	2.0	0.6	12663				
2	1	0	0	0	1553		1655	6622	7839
2	1	0	0	0	2059		2195	8780	
2	1	0	0	0	1973		2103	8413	
2	1	0	0	0	1768		1884	7539	
2	2	10	0	0	1181		1259	5036	4965
2	2	10	0	0	1003		1069	4277	
2	2	10	0	0	1282		1366	5467	
2	2	10	0	0	1191		1269	5079	

2	3	10	0.5	0	991	1056	4226	5446
2	3	10	0.5	0	1606	1712	6849	
2	3	10	0.5	0	1459	1555	6222	
2	3	10	0.5	0	1052	1121	4486	
2	4	10	2.0	0	2438	2599	10398	9369
2	4	10	2.0	0	1912	2038	8155	
2	4	10	2.0	0	2379	2536	10147	
2	4	10	2.0	0	2058	2194	8777	
2	5	10	0	0.06	1261	1344	5379	5563
2	5	10	0	0.06	1500	1599	6399	
2	5	10	0	0.06	1432	1527	6109	
2	5	10	0	0.06	1023	1091	4364	
2	6	10	0.5	0.06	1037	1106	4424	6171
2	6	10	0.5	0.06	1527	1628	6514	
2	6	10	0.5	0.06	1591	1696	6787	
2	6	10	0.5	0.06	1631	1739	6958	
2	7	10	2.0	0.06	1299	1385	5542	7833
2	7	10	2.0	0.06	2471	2634	10538	
2	7	10	2.0	0.06	1631	1739	6959	
2	7	10	2.0	0.06	1945	2073	8294	
2	8	10	0	0.6	1737	1852	7408	7209
2	8	10	0	0.6	1707	1820	7280	
2	8	10	0	0.6	1475	1572	6291	
2	8	10	0	0.6	1842	1964	7856	
2	9	10	0.5	0.6	1621	1728	6914	7117
2	9	10	0.5	0.6	1529	1630	6522	
2	9	10	0.5	0.6	1934	2062	8249	
2	9	10	0.5	0.6	1590	1695	6782	
2	10	10	2.0	0.6	2323	2477	9909	8337
2	10	10	2.0	0.6	1715	1829	7316	
2	10	10	2.0	0.6	1581	1686	6744	
2	10	10	2.0	0.6	2199	2345	9381	
3	1	0	0	0	7770	7961	31844	22733
3	1	0	0	0	5094	5219	20877	
3	1	0	0	0	4528	4639	18557	
3	1	0	0	0	4796	4914	19656	
3	2	10	0	0	1669	1710	6840	9368
3	2	10	0	0	2109	2160	8643	
3	2	10	0	0	2509	2570	10283	
3	2	10	0	0	2856	2926	11705	
3	3	10	0.5	0	5413	5546	22186	11260
3	3	10	0.5	0	1450	1485	5943	
3	3	10	0.5	0	2064	2115	8460	
3	3	10	0.5	0	2062	2112	8451	
3	4	10	2.0	0	3926	4023	16093	13639
3	4	10	2.0	0	3059	3134	12539	

3	4	10	2.0	0	3661	3751	15007	
3	4	10	2.0	0	2664	2730	10920	
3	5	10	0	0.06	2468	2529	10118	9806
3	5	10	0	0.06	2345	2403	9614	
3	5	10	0	0.06	2440	2500	10000	
3	5	10	0	0.06	2315	2372	9491	
3	6	10	0.5	0.06	2217	2271	9086	10041
3	6	10	0.5	0.06	2379	2437	9750	
3	6	10	0.5	0.06	2755	2823	11292	
3	6	10	0.5	0.06	2449	2509	10037	
3	7	10	2.0	0.06	3653	3743	14975	14485
3	7	10	2.0	0.06	2855	2925	11703	
3	7	10	2.0	0.06	2922	2994	11977	
3	7	10	2.0	0.06	4705	4821	19285	
3	8	10	0	0.6	3592	3681	14724	13544
3	8	10	0	0.6	3373	3456	13826	
3	8	10	0	0.6	2947	3020	12080	
3	8	10	0	0.6				
3	9	10	0.5	0.6	4122	4223	16894	13987
3	9	10	0.5	0.6	3389	3472	13889	
3	9	10	0.5	0.6	3138	3216	12864	
3	9	10	0.5	0.6	3001	3075	12303	
3	10	10	2.0	0.6				25584
3	10	10	2.0	0.6	6011	6159	24636	
3	10	10	2.0	0.6	6841	7009	28039	
3	10	10	2.0	0.6	5875	6019	24078	
4	1	0	0	0	2510	2653	10613	12166
4	1	0	0	0	2636	2786	11146	
4	1	0	0	0	3316	3505	14021	
4	1	0	0	0	3047	3221	12884	
4	2	10	0	0	2214	2340	9361	10606
4	2	10	0	0	2447	2586	10347	
4	2	10	0	0	3443	3639	14558	
4	2	10	0	0	1929	2039	8156	
4	3	10	0.5	0	1821	1925	7700	14491
4	3	10	0.5	0	2448	2588	10352	
4	3	10	0.5	0	3985	4213	16852	
4	3	10	0.5	0	5453	5765	23060	
4	4	10	2.0	0	6403	6769	27076	17205
4	4	10	2.0	0	2988	3158	12635	
4	4	10	2.0	0	3730	3943	15773	
4	4	10	2.0	0	3154	3334	13337	
4	5	10	0	0.06	3863	4084	16337	17928
4	5	10	0	0.06	5415	5724	22896	
4	5	10	0	0.06	3441	3638	14552	
4	5	10	0	0.06				

4	6	10	0.5	0.06	3402	3596	14385	13860
4	6	10	0.5	0.06	3721	3933	15734	
4	6	10	0.5	0.06	3106	3283	13133	
4	6	10	0.5	0.06	2883	3047	12190	
4	7	10	2.0	0.06	3559	3762	15048	19989
4	7	10	2.0	0.06	2667	2820	11280	
4	7	10	2.0	0.06	6878	7270	29082	
4	7	10	2.0	0.06	5804	6136	24544	
4	8	10	0	0.6	3933	4158	16632	16252
4	8	10	0	0.6	3377	3570	14281	
4	8	10	0	0.6	2968	3137	12551	
4	8	10	0	0.6	5094	5385	21542	
4	9	10	0.5	0.6	4598	4860	19442	15655
4	9	10	0.5	0.6	3208	3392	13568	
4	9	10	0.5	0.6	4119	4354	17416	
4	9	10	0.5	0.6	2883	3048	12193	
4	10	10	2.0	0.6	3843	4063	16253	17854
4	10	10	2.0	0.6	3169	3350	13402	
4	10	10	2.0	0.6	4532	4790	19163	
4	10	10	2.0	0.6	5344	5649	22597	
5	1	0	0	0	4364	4471	17885	22918
5	1	0	0	0	4883	5003	20012	
5	1	0	0	0	5084	5209	20836	
5	1	0	0	0	8037	8234	32939	
5	2	10	0	0	4903	5023	20095	21660
5	2	10	0	0	5420	5553	22214	
5	2	10	0	0	7073	7247	28989	
5	2	10	0	0	3744	3836	15345	
5	3	10	0.5	0	4525	4636	18547	19638
5	3	10	0.5	0	3857	3952	15809	
5	3	10	0.5	0	4703	4819	19277	
5	3	10	0.5	0	6080	6230	24921	
5	4	10	2.0	0	8134	8334	33338	41792
5	4	10	2.0	0	9276	9504	38019	
5	4	10	2.0	0	10742	11006	44024	
5	4	10	2.0	0	12636	12947	51788	
5	5	10	0	0.06	4163	4266	17064	22567
5	5	10	0	0.06	5259	5388	21553	
5	5	10	0	0.06	6520	6680	26722	
5	5	10	0	0.06	6082	6231	24927	
5	6	10	0.5	0.06	7388	7570	30280	24931
5	6	10	0.5	0.06	6400	6557	26230	
5	6	10	0.5	0.06	5391	5524	22097	
5	6	10	0.5	0.06	5152	5279	21118	
5	7	10	2.0	0.06	7126	7301	29205	29591
5	7	10	2.0	0.06	6278	6433	25733	

5	7	10	2.0	0.06	7939		8134	32538	
5	7	10	2.0	0.06	7536		7721	30886	
5	8	10	0	0.6	7848		8041	32164	28529
5	8	10	0	0.6	8628		8840	35362	
5	8	10	0	0.6	5303		5434	21737	
5	8	10	0	0.6	6064		6213	24852	
5	9	10	0.5	0.6	5685		5825	23301	34610
5	9	10	0.5	0.6	6107		6257	25301	
5	9	10	0.5	0.6					
5	9	10	0.5	0.6	13541		13874	55499	
5	10	10	2.0	0.6	7673		7862	31449	35184
5	10	10	2.0	0.6	10157		10407	41628	
5	10	10	2.0	0.6	8645		8857	35430	
5	10	10	2.0	0.6	7863		8057	32229	
6	1	0	0	0	5578	5213	5779	23117	22801
6	1	0	0	0	4847				
6	1	0	0	0	4147	4216	4674	18698	
6	1	0	0	0	4285				
6	1	0	0	0	5278	5995	6647	26588	
6	1	0	0	0	6713				
6	1	0	0	0					
6	1	0	0	0					
6	2	10	0	0	3817	3815	4229	16919	17829
6	2	10	0	0	3812				
6	2	10	0	0	3822	3972	4403	17614	
6	2	10	0	0	4121				
6	2	10	0	0	4305	4356	4829	19319	
6	2	10	0	0	4407				
6	2	10	0	0	3819	3938	4366	17466	
6	2	10	0	0	4057				
6	3	10	0.5	0	2407	2687	2979	11916	10205
6	3	10	0.5	0	2967				
6	3	10	0.5	0	1503	1745	1935	7742	
6	3	10	0.5	0	1988				
6	3	10	0.5	0	2335	2811	3116	12467	
6	3	10	0.5	0	3287				
6	3	10	0.5	0	1856	1960	2173	8695	
6	3	10	0.5	0	2064				
6	4	10	2.0	0	6882	7050	7816	31265	27478
6	4	10	2.0	0	7218				
6	4	10	2.0	0	5897	6146	6814	27258	
6	4	10	2.0	0	6395				
6	4	10	2.0	0	7223	7386	8189	32758	
6	4	10	2.0	0	7550				
6	4	10	2.0	0	4139	4201	4658	18633	
6	4	10	2.0	0	4263				

6	5	10	0	0.06	6361	6863	7609	30436	28873
6	5	10	0	0.06	7365				
6	5	10	0	0.06	8908	8133	9016	36067	
6	5	10	0	0.06	7357				
6	5	10	0	0.06	4147	5417	6005	24022	
6	5	10	0	0.06	6686				
6	5	10	0	0.06	5437	5629	6241	24966	
6	5	10	0	0.06	5822				
6	6	10	0.5	0.06	4022	3706	4108	16435	17732
6	6	10	0.5	0.06	3390				
6	6	10	0.5	0.06	4491	3613	4005	16022	
6	6	10	0.5	0.06	2735				
6	6	10	0.5	0.06	4243	4070	4512	18051	
6	6	10	0.5	0.06	3897				
6	6	10	0.5	0.06	4165	4605	5105	20421	
6	6	10	0.5	0.06	5044				
6	7	10	2.0	0.06	4516	4583	5081	20326	23579
6	7	10	2.0	0.06	4650				
6	7	10	2.0	0.06	3950	4037	4476	17906	
6	7	10	2.0	0.06	4125				
6	7	10	2.0	0.06	4585	4756	5272	21091	
6	7	10	2.0	0.06	4926				
6	7	10	2.0	0.06	7690	7890	8747	34991	
6	7	10	2.0	0.06	8091				
6	8	10	0	0.6	8928	9795	10860	43440	32884
6	8	10	0	0.6	10663				
6	8	10	0	0.6	6360	6119	6784	27137	
6	8	10	0	0.6	5878				
6	8	10	0	0.6	6555	6859	7604	30418	
6	8	10	0	0.6	7162				
6	8	10	0	0.6	6874	6886	7635	30540	
6	8	10	0	0.6	6899				
6	9	10	0.5	0.6	5298	5621	6232	24928	21534
6	9	10	0.5	0.6	5944				
6	9	10	0.5	0.6	5995	6426	7124	28498	
6	9	10	0.5	0.6	6857				
6	9	10	0.5	0.6	1736	1653	1833	7332	
6	9	10	0.5	0.6	1570				
6	9	10	0.5	0.6	5516	5723	6345	25380	
6	9	10	0.5	0.6	5929				
6	10	10	2.0	0.6	5946	6500	7207	28829	24671
6	10	10	2.0	0.6	7055				
6	10	10	2.0	0.6	4205	4272	4736	18946	
6	10	10	2.0	0.6	4339				
6	10	10	2.0	0.6	5739	5854	6490	25960	
6	10	10	2.0	0.6	5968				

6	10	10	2.0	0.6	5656	5625	6237	24948	
6	10	10	2.0	0.6	5595				
7	1	0	0	0	6000		6191	24767	16211
7	1	0	0	0	3247		3350	13403	
7	1	0	0	0	3898		4022	16091	
7	1	0	0	0	2564		2646	10584	
7	2	10	0	0	2118		2185	8743	10042
7	2	10	0	0	3438		3548	14192	
7	2	10	0	0	2060		2126	8504	
7	2	10	0	0	2115		2182	8731	
7	3	10	0.5	0	1856		1915	7662	12813
7	3	10	0.5	0	1950		2012	8050	
7	3	10	0.5	0	5658		5839	23359	
7	3	10	0.5	0	2951		3045	12183	
7	4	10	2.0	0	3055		3152	12611	19046
7	4	10	2.0	0	3765		3885	15543	
7	4	10	2.0	0	2450		2528	10114	
7	4	10	2.0	0	9184		9478	37915	
7	5	10	0	0.06	5569		5747	22988	20215
7	5	10	0	0.06	4927		5085	20342	
7	5	10	0	0.06	2861		2953	11812	
7	5	10	0	0.06	6230		6429	25718	
7	6	10	0.5	0.06	3151		3251	13007	15999
7	6	10	0.5	0.06	3966		4093	16372	
7	6	10	0.5	0.06	4610		4758	19032	
7	6	10	0.5	0.06	3775		3896	15584	
7	7	10	2.0	0.06	3167		3269	13077	15262
7	7	10	2.0	0.06	5335		5506	22025	
7	7	10	2.0	0.06	2494		2574	10298	
7	7	10	2.0	0.06	3791		3912	15650	
7	8	10	0	0.6	2862		2954	11816	10977
7	8	10	0	0.6	2769		2858	11432	
7	8	10	0	0.6	2346		2421	9685	
7	8	10	0	0.6					
7	9	10	0.5	0.6	2664		2749	10999	14913
7	9	10	0.5	0.6	2867		2959	11838	
7	9	10	0.5	0.6	5409		5582	22330	
7	9	10	0.5	0.6	3509		3621	14485	
7	10	10	2.0	0.6	6399		6604	26416	17660
7	10	10	2.0	0.6	3393		3502	14009	
7	10	10	2.0	0.6	3750		3870	15483	
7	10	10	2.0	0.6	3568		3683	14732	

APPENDIX B. ALCIAN BLUE MEDIA DATA

A Horse	Tx Group	IL-1 ug/mL	HA mg/mL	TA mg/mL	Raw Data	Avg for replicate	Convert Decay	Total Media	Avg for tx group
1	1	0	0	0	2601	2571	2613	52260	26537
1	1	0	0	0	2541				
1	1	0	0	0	2772	2746	2791	55828	
1	1	0	0	0	2721				
1	1	0	0	0	2112	2130	2164	43297	
1	1	0	0	0	2148				
1	1	0	0	0	3012	2996	3045	60912	
1	1	0	0	0	2981				
1	2	10	0	0	2444	2395	2434	48696	19918
1	2	10	0	0	2347				
1	2	10	0	0	1736	1562	1587	31755	
1	2	10	0	0	1388				
1	2	10	0	0	2318	2207	2242	44859	
1	2	10	0	0	2095				
1	2	10	0	0	1787	1674	1701	34037	
1	2	10	0	0	1564				
1	3	10	0.5	0					
1	3	10	0.5	0					
1	3	10	0.5	0					
1	3	10	0.5	0					
1	3	10	0.5	0					
1	3	10	0.5	0					
1	3	10	0.5	0					
1	3	10	0.5	0					
1	4	10	2.0	0	1420	1476	1500	30006	12856
1	4	10	2.0	0	1532				
1	4	10	2.0	0	775	748	760	15207	
1	4	10	2.0	0	721				
1	4	10	2.0	0	1244	1017	1034	20686	
1	4	10	2.0	0	791				
1	4	10	2.0	0	1424	1818	1847	36951	
1	4	10	2.0	0	2211				
1	5	10	0	0.06	4360	4115	4182	83642	29364
1	5	10	0	0.06	3869				
1	5	10	0	0.06	4487	3638	3698	73963	
1	5	10	0	0.06	2790				
1	5	10	0	0.06	2228	1985	2018	40363	
1	5	10	0	0.06	1743				
1	5	10	0	0.06	1633	1817	1847	36947	
1	5	10	0	0.06	2002				
1	6	10	0.5	0.06					
1	6	10	0.5	0.06					

1	6	10	0.5	0.06					
1	6	10	0.5	0.06					
1	6	10	0.5	0.06					
1	6	10	0.5	0.06					
1	6	10	0.5	0.06					
1	6	10	0.5	0.06					
1	7	10	2.0	0.06	1692	1409	1432	28643	23688
1	7	10	2.0	0.06	1126				
1	7	10	2.0	0.06	2623	2507	2548	50968	
1	7	10	2.0	0.06	2392				
1	7	10	2.0	0.06	1042	1099	1117	22345	
1	7	10	2.0	0.06	1156				
1	7	10	2.0	0.06	6090	4307	4377	87552	
1	7	10	2.0	0.06	2524				
1	8	10	0	0.6	1375	1547	1572	31459	17368
1	8	10	0	0.6	1719				
1	8	10	0	0.6	1699	1987	2019	40397	
1	8	10	0	0.6	2275				
1	8	10	0	0.6	2009	1971	2003	40062	
1	8	10	0	0.6	1933				
1	8	10	0	0.6	1213	1329	1351	27027	
1	8	10	0	0.6	1445				
1	9	10	0.5	0.6					
1	9	10	0.5	0.6					
1	9	10	0.5	0.6					
1	9	10	0.5	0.6					
1	9	10	0.5	0.6					
1	9	10	0.5	0.6					
1	9	10	0.5	0.6					
1	9	10	0.5	0.6					
1	10	10	2.0	0.6	445	406	412	8259	18184
1	10	10	2.0	0.6	367				
1	10	10	2.0	0.6	4647	4119	4186	83729	
1	10	10	2.0	0.6	3591				
1	10	10	2.0	0.6	1831	1874	1905	38106	
1	10	10	2.0	0.6	1917				
1	10	10	2.0	0.6	711	756	769	15381	
1	10	10	2.0	0.6	801				
2	1	0	0	0	446		475	9516	9847
2	1	0	0	0	385		410	8215	
2	1	0	0	0	473		504	10092	
2	1	0	0	0	542		578	11565	
2	2	10	0	0	275		293	5868	8379
2	2	10	0	0	422		450	9005	
2	2	10	0	0	481		513	10264	
2	2	10	0	0					

2	3	10	0.5	0	3230	3444	68886	69513
2	3	10	0.5	0				
2	3	10	0.5	0	3946	4207	84146	
2	3	10	0.5	0	2603	2775	55507	
2	4	10	2.0	0	1113	1186	23731	19572
2	4	10	2.0	0	754	803	16077	
2	4	10	2.0	0	886	945	18909	
2	4	10	2.0	0				
2	5	10	0	0.06	449	479	9584	12615
2	5	10	0	0.06	605	645	12914	
2	5	10	0	0.06	763	814	16287	
2	5	10	0	0.06	547	583	11676	
2	6	10	0.5	0.06	297	317	6340	6222
2	6	10	0.5	0.06	268	286	5721	
2	6	10	0.5	0.06	254	271	5422	
2	6	10	0.5	0.06	347	370	7407	
2	7	10	2.0	0.06	218	232	4654	8395
2	7	10	2.0	0.06	222	236	4739	
2	7	10	2.0	0.06	820	874	17485	
2	7	10	2.0	0.06	314	335	6703	
2	8	10	0	0.6	489	521	10436	9289
2	8	10	0	0.6	611	652	13040	
2	8	10	0	0.6	310	330	6616	
2	8	10	0	0.6	331	353	7064	
2	9	10	0.5	0.6	492	525	10501	8521
2	9	10	0.5	0.6	437	466	9327	
2	9	10	0.5	0.6	383	408	8174	
2	9	10	0.5	0.6	285	304	6083	
2	10	10	2.0	0.6	398	424	8495	8298
2	10	10	2.0	0.6	253	270	5400	
2	10	10	2.0	0.6	401	427	8559	
2	10	10	2.0	0.6	503	536	10737	
3	1	0	0	0	319	327	6542	10111
3	1	0	0	0	612	627	12551	
3	1	0	0	0	506	518	10377	
3	1	0	0	0	535	548	10972	
3	2	10	0	0	275	282	5640	6045
3	2	10	0	0	481	493	9865	
3	2	10	0	0	215	220	4409	
3	2	10	0	0	208	213	4266	
3	3	10	0.5	0	458	469	9394	10989
3	3	10	0.5	0	672	689	13783	
3	3	10	0.5	0	464	475	9517	
3	3	10	0.5	0	549	563	11261	
3	4	10	2.0	0	432	443	8861	8779
3	4	10	2.0	0	373	382	7651	

3	4	10	2.0	0	479	491	9825	
3	4	10	2.0	0				
3	5	10	0	0.06	225	230	4616	3929
3	5	10	0	0.06	211	216	4329	
3	5	10	0	0.06	131	134	2687	
3	5	10	0	0.06	199	204	4082	
3	6	10	0.5	0.06	800	819	16394	11090
3	6	10	0.5	0.06	608	623	12475	
3	6	10	0.5	0.06	302	309	6196	
3	6	10	0.5	0.06	453	464	9295	
3	7	10	2.0	0.06	534	547	10957	13261
3	7	10	2.0	0.06	776	795	15903	
3	7	10	2.0	0.06	851	872	17442	
3	7	10	2.0	0.06	426	437	8741	
3	8	10	0	0.6	318	326	6523	5384
3	8	10	0	0.6	239	245	4902	
3	8	10	0	0.6	246	252	5046	
3	8	10	0	0.6	247	253	5067	
3	9	10	0.5	0.6	539	552	11057	11037
3	9	10	0.5	0.6				
3	9	10	0.5	0.6	559	573	11468	
3	9	10	0.5	0.6	516	529	10586	
3	10	10	2.0	0.6	548	562	11243	9807
3	10	10	2.0	0.6	356	365	7303	
3	10	10	2.0	0.6	633	649	12987	
3	10	10	2.0	0.6	375	384	7693	
4	1	0	0	0	731	785	3140	3596
4	1	0	0	0	861	924	3699	
4	1	0	0	0	775	832	3329	
4	1	0	0	0	981	1053	4214	
4	2	10	0	0	625	671	2685	4303
4	2	10	0	0	1068	1147	4588	
4	2	10	0	0	542	582	2328	
4	2	10	0	0	1771	1902	7609	
4	3	10	0.5	0	1780	1912	7648	4132
4	3	10	0.5	0	692	743	2973	
4	3	10	0.5	0	617	662	2651	
4	3	10	0.5	0	758	814	3257	
4	4	10	2.0	0	1496	1606	6427	8702
4	4	10	2.0	0	1630	1750	7003	
4	4	10	2.0	0	2492	2676	10707	
4	4	10	2.0	0	2483	2667	10669	
4	5	10	0	0.06	683	733	2935	2810
4	5	10	0	0.06	748	803	3214	
4	5	10	0	0.06	585	628	2513	
4	5	10	0	0.06	600	644	2578	

4	6	10	0.5	0.06	368	395	1581	1778
4	6	10	0.5	0.06	409	439	1757	
4	6	10	0.5	0.06	423	454	1818	
4	6	10	0.5	0.06	455	488	1955	
4	7	10	2.0	0.06	2042	2193	8775	5952
4	7	10	2.0	0.06	1894	2034	8139	
4	7	10	2.0	0.06	755	811	3244	
4	7	10	2.0	0.06	849	912	3648	
4	8	10	0	0.6	604	648	2595	2724
4	8	10	0	0.6				
4	8	10	0	0.6	864	928	3712	
4	8	10	0	0.6	434	466	1865	
4	9	10	0.5	0.6	534	573	2294	2192
4	9	10	0.5	0.6	572	614	2548	
4	9	10	0.5	0.6	406	436	1744	
4	9	10	0.5	0.6	529	568	2273	
4	10	10	2.0	0.6	914	981	3927	7290
4	10	10	2.0	0.6	1549	1664	6656	
4	10	10	2.0	0.6	1300	1396	5586	
4	10	10	2.0	0.6	3023	3247	12991	
5	1	0	0	0	817	837	3349	4546
5	1	0	0	0	751	769	3079	
5	1	0	0	0	1990	2039	8158	
5	1	0	0	0	878	899	3599	
5	2	10	0	0	625	640	2562	2738
5	2	10	0	0	603	618	2472	
5	2	10	0	0	881	903	3612	
5	2	10	0	0	563	577	2308	
5	3	10	0.5	0	720	738	2952	3346
5	3	10	0.5	0	1095	1122	4489	
5	3	10	0.5	0	591	605	2423	
5	3	10	0.5	0	859	880	3522	
5	4	10	2.0	0	2642	2707	10828	11152
5	4	10	2.0	0	1865	1911	7646	
5	4	10	2.0	0	1186	1215	4862	
5	4	10	2.0	0	5190	5318	21272	
5	5	10	0	0.06	1086	1113	4453	4597
5	5	10	0	0.06	1098	1125	4502	
5	5	10	0	0.06	1102	1129	4518	
5	5	10	0	0.06	1199	1229	4916	
5	6	10	0.5	0.06	1421	1456	5827	5084
5	6	10	0.5	0.06	1037	1063	4252	
5	6	10	0.5	0.06	1008	1033	4134	
5	6	10	0.5	0.06	1494	1530	6123	
5	7	10	2.0	0.06	4466	4576	18307	14994
5	7	10	2.0	0.06	2494	2555	10223	

5	7	10	2.0	0.06	3351		3433	13734	
5	7	10	2.0	0.06	4321		4428	17712	
5	8	10	0	0.6	801		821	3284	3553
5	8	10	0	0.6	783		802	3210	
5	8	10	0	0.6	1017		1042	4170	
5	8	10	0	0.6	865		886	3546	
5	9	10	0.5	0.6	962		986	3945	3775
5	9	10	0.5	0.6	1019		1044	4178	
5	9	10	0.5	0.6	989		920	3682	
5	9	10	0.5	0.6	804		824	3297	
5	10	10	2.0	0.6	3831		3925	15701	10626
5	10	10	2.0	0.6	1987		2036	8144	
5	10	10	2.0	0.6	2603		2667	10670	
5	10	10	2.0	0.6	1949		1997	7988	
6	1	0	0	0	947	982	1089	4356	4703
6	1	0	0	0	1016				
6	1	0	0	0	815	920	1020	4080	
6	1	0	0	0	1024				
6	1	0	0	0	1315	1280	1419	5678	
6	1	0	0	0	1245				
6	1	0	0	0	1412	1059	1174	4698	
6	1	0	0	0	706				
6	2	10	0	0	702	736	816	3265	4536
6	2	10	0	0	770				
6	2	10	0	0	1009	1241	1376	5505	
6	2	10	0	0	1473				
6	2	10	0	0	976	1091	1210	4841	
6	2	10	0	0	1206				
6	2	10	0	0	1183	1022	1133	4532	
6	2	10	0	0	860				
6	3	10	0.5	0	539	613	680	2721	2599
6	3	10	0.5	0	688				
6	3	10	0.5	0	647	770	853	3415	
6	3	10	0.5	0	892				
6	3	10	0.5	0	390	384	426	1704	
6	3	10	0.5	0	377				
6	3	10	0.5	0	481	576	638	2555	
6	3	10	0.5	0	671				
6	4	10	2.0	0	696	757	839	3357	6064
6	4	10	2.0	0	817				
6	4	10	2.0	0	1832	1893	2099	8398	
6	4	10	2.0	0	1955				
6	4	10	2.0	0	806	883	979	3919	
6	4	10	2.0	0	961				
6	4	10	2.0	0	1953	1935	2145	8581	
6	4	10	2.0	0	1917				

6	5	10	0	0.06	624	655	726	2906	3374
6	5	10	0	0.06	686				
6	5	10	0	0.06	743	660	732	2929	
6	5	10	0	0.06	577				
6	5	10	0	0.06	788	756	838	3353	
6	5	10	0	0.06	723				
6	5	10	0	0.06	1144	971	1076	4307	
6	5	10	0	0.06	797				
6	6	10	0.5	0.06	517	536	594	2378	2191
6	6	10	0.5	0.06	555				
6	6	10	0.5	0.06	447	464	515	2060	
6	6	10	0.5	0.06	482				
6	6	10	0.5	0.06	469	451	500	2000	
6	6	10	0.5	0.06	433				
6	6	10	0.5	0.06	536	524	581	2327	
6	6	10	0.5	0.06	513				
6	7	10	2.0	0.06	1193	1355	1502	6010	3816
6	7	10	2.0	0.06	1516				
6	7	10	2.0	0.06	354	350	388	1555	
6	7	10	2.0	0.06	346				
6	7	10	2.0	0.06	913	919	1019	4078	
6	7	10	2.0	0.06	926				
6	7	10	2.0	0.06	782	817	905	3623	
6	7	10	2.0	0.06	851				
6	8	10	0	0.6	667	667	739	2959	3851
6	8	10	0	0.6					
6	8	10	0	0.6	681	941	1043	4173	
6	8	10	0	0.6	1200				
6	8	10	0	0.6	874	906	1004	4017	
6	8	10	0	0.6	937				
6	8	10	0	0.6	979	959	1063	4255	
6	8	10	0	0.6	940				
6	9	10	0.5	0.6	855	827	916	3667	2664
6	9	10	0.5	0.6	798				
6	9	10	0.5	0.6	609	588	652	2609	
6	9	10	0.5	0.6	567				
6	9	10	0.5	0.6	782	833	924	3696	
6	9	10	0.5	0.6	884				
6	9	10	0.5	0.6	137	153	170	682	
6	9	10	0.5	0.6	170				
6	10	10	2.0	0.6	1061	1061	1176	4707	4055
6	10	10	2.0	0.6	1061				
6	10	10	2.0	0.6	914	1049	1163	4653	
6	10	10	2.0	0.6	1184				
6	10	10	2.0	0.6	986	977	1083	4333	
6	10	10	2.0	0.6	968				

6	10	10	2.0	0.6	569	569	631	2527	
6	10	10	2.0	0.6	570				
7	1	0	0	0	909		938	3755	3659
7	1	0	0	0	958		989	3958	
7	1	0	0	0	823		850	3400	
7	1	0	0	0	853		881	3524	
7	2	10	0	0	665		868	2747	3256
7	2	10	0	0	832		859	3437	
7	2	10	0	0	830		857	3429	
7	2	10	0	0	826		853	3412	
7	3	10	0.5	0	681		703	2813	2563
7	3	10	0.5	0	582		601	2404	
7	3	10	0.5	0	779		804	3218	
7	3	10	0.5	0	440		454	1818	
7	4	10	2.0	0	604		623	2495	5681
7	4	10	2.0	0	307		317	1268	
7	4	10	2.0	0	4272		4409	17363	
7	4	10	2.0	0	321		331	1326	
7	5	10	0	0.06	870		898	3595	2966
7	5	10	0	0.06	632		652	2611	
7	5	10	0	0.06	793		819	3276	
7	5	10	0	0.06	576		595	2380	
7	6	10	0.5	0.06	536		553	2215	2278
7	6	10	0.5	0.06	461		476	1905	
7	6	10	0.5	0.06	667		689	2756	
7	6	10	0.5	0.06	541		558	2235	
7	7	10	2.0	0.06	1052		1085	4343	2219
7	7	10	2.0	0.06	391		403	1615	
7	7	10	2.0	0.06	323		333	1334	
7	7	10	2.0	0.06	383		395	1582	
7	8	10	0	0.6	418		431	1727	1948
7	8	10	0	0.6	361		372	1491	
7	8	10	0	0.6	526		543	2173	
7	8	10	0	0.6	581		600	2400	
7	9	10	0.5	0.6	506		522	2091	1703
7	9	10	0.5	0.6	452		466	1867	
7	9	10	0.5	0.6	377		389	1558	
7	9	10	0.5	0.6	314		324	1297	
7	10	10	2.0	0.6	338		349	1396	1415
7	10	10	2.0	0.6	436		450	1801	
7	10	10	2.0	0.6	254		262	1049	
7	10	10	2.0	0.6	342		353	1413	

APPENDIX C. DMMB PELLET DATA

Horse	Tx Group	IL-1 ug/mL	HA mg/mL	TA mg/mL	Optical Density	Chondr. ug/mL	Total/ Pellet	Avg for tx group
1	1	0	0	0	0.4429	25.69	5.13	5.93
1	1	0	0	0	0.4832	31.89	6.37	
1	1	0	0	0	0.4762	30.81	6.16	
1	1	0	0	0	0.4722	30.20	6.04	
1	2	10	0	0	0.4387	25.04	5.01	5.18
1	2	10	0	0	0.4127	21.04	4.20	
1	2	10	0	0	0.4517	27.04	5.40	
1	2	10	0	0	0.4745	30.55	6.11	
1	3	10	0.5	0				
1	3	10	0.5	0				
1	3	10	0.5	0				
1	3	10	0.5	0				
1	4	10	2.0	0	0.5238	38.13	7.62	6.66
1	4	10	2.0	0	0.4895	32.86	6.57	
1	4	10	2.0	0	0.4857	32.27	6.45	
1	4	10	2.0	0	0.4713	30.06	6.01	
1	5	10	0	0.06	0.4712	30.04	6.00	6.82
1	5	10	0	0.06	0.5002	34.50	6.90	
1	5	10	0	0.06	0.5051	35.26	7.05	
1	5	10	0	0.06	0.5144	36.69	7.33	
1	6	10	0.5	0.06				
1	6	10	0.5	0.06				
1	6	10	0.5	0.06				
1	6	10	0.5	0.06				
1	7	10	2.0	0.06	0.4556	27.64	5.52	5.97
1	7	10	2.0	0.06	0.4960	33.86	6.77	
1	7	10	2.0	0.06	0.4524	27.15	5.43	
1	7	10	2.0	0.06	0.4764	30.84	6.16	
1	8	10	0	0.6	0.4815	31.63	6.32	6.04
1	8	10	0	0.6	0.4604	28.38	5.67	
1	8	10	0	0.6	0.4869	32.46	6.49	
1	8	10	0	0.6	0.4609	28.46	5.69	
1	9	10	0.5	0.6				
1	9	10	0.5	0.6				
1	9	10	0.5	0.6				
1	9	10	0.5	0.6				
1	10	10	2.0	0.6	0.4436	25.80	5.16	5.85
1	10	10	2.0	0.6	0.3892	17.43		
1	10	10	2.0	0.6	0.4736	30.41	6.08	
1	10	10	2.0	0.6	0.4810	31.55	6.31	
2	1	0	0	0	0.3648	13.67	2.73	2.47
2	1	0	0	0	0.3586	12.72	2.54	

2	1	0	0	0	0.3456	10.72	2.14	
2	1	0	0	0	0.3561	12.33	2.46	
2	2	10	0	0	0.3539	12.00	2.40	2.40
2	2	10	0	0	0.3571	12.49	2.49	
2	2	10	0	0	0.3466	10.87	2.17	
2	2	10	0	0	0.3585	12.70	2.54	
2	3	10	0.5	0	0.3615	13.16	2.63	2.65
2	3	10	0.5	0	0.3696	14.41	2.88	
2	3	10	0.5	0	0.3584	12.69	2.53	
2	3	10	0.5	0	0.3589	12.76	2.55	
2	4	10	2.0	0	0.5099	36.00	7.20	6.38
2	4	10	2.0	0	0.4387	25.04	5.00	
2	4	10	2.0	0	0.5088	35.83	7.16	
2	4	10	2.0	0	0.4767	30.89	6.17	
2	5	10	0	0.06	0.3335	8.86	1.77	2.36
2	5	10	0	0.06	0.3373	9.44	1.88	
2	5	10	0	0.06	0.4032	19.58	3.91	
2	5	10	0	0.06	0.3376	9.49	1.89	
2	6	10	0.5	0.06	0.3626	13.33	2.66	2.78
2	6	10	0.5	0.06	0.3659	13.84	2.76	
2	6	10	0.5	0.06	0.3642	13.58	2.71	
2	6	10	0.5	0.06	0.3732	14.96	2.99	
2	7	10	2.0	0.06	0.4024	19.46	3.89	4.75
2	7	10	2.0	0.06	0.4631	28.8	5.76	
2	7	10	2.0	0.06	0.4152	21.43	4.28	
2	7	10	2.0	0.06	0.4409	25.38	5.07	
2	8	10	0	0.6	0.4125	21.01	4.20	4.07
2	8	10	0	0.6	0.3989	18.92	3.78	
2	8	10	0	0.6	0.4205	22.24	4.44	
2	8	10	0	0.6	0.4009	19.23	3.84	
2	9	10	0.5	0.6	0.5218	37.83	7.56	5.04
2	9	10	0.5	0.6	0.3817	16.27	3.25	
2	9	10	0.5	0.6	0.4785	31.16	6.23	
2	9	10	0.5	0.6	0.3775	15.63	3.12	
2	10	10	2.0	0.6	0.4647	29.04	5.80	6.54
2	10	10	2.0	0.6	0.5263	38.52	7.70	
2	10	10	2.0	0.6	0.4885	32.70	6.54	
2	10	10	2.0	0.6	0.4749	3.61	6.12	
3	1	0	0	0	0.3818	15.17	3.03	3.03
3	1	0	0	0	0.3516	10.79	2.15	
3	1	0	0	0	0.3950	17.08	3.41	
3	1	0	0	0	0.3983	17.56	3.51	
3	2	10	0	0	0.3424	9.46	1.98	2.22
3	2	10	0	0	0.3364	8.59	1.71	
3	2	10	0	0	0.3681	13.18	2.63	
3	2	10	0	0	0.3679	13.15	2.63	

3	3	10	0.5	0	0.4378	23.28	4.65	3.62
3	3	10	0.5	0				
3	3	10	0.5	0	0.3660	12.88	2.57	
3	3	10	0.5	0	0.4032	18.27	3.65	
3	4	10	2.0	0	0.4081	18.98	3.79	3.97
3	4	10	2.0	0	0.4411	23.76	4.75	
3	4	10	2.0	0	0.4195	20.63	4.12	
3	4	10	2.0	0	0.3876	16.01	3.20	
3	5	10	0	0.06	0.3374	8.73	1.74	1.97
3	5	10	0	0.06	0.3534	11.05	2.21	
3	5	10	0	0.06	0.3468	10.10	2.02	
3	5	10	0	0.06	0.3438	9.66	1.93	
3	6	10	0.5	0.06	0.3648	12.71	2.54	2.64
3	6	10	0.5	0.06	0.3626	12.39	2.47	
3	6	10	0.5	0.06	0.3704	13.52	2.70	
3	6	10	0.5	0.06	0.3760	14.33	2.86	
3	7	10	2.0	0.06	0.4169	20.26	4.05	4.17
3	7	10	2.0	0.06	0.4024	18.15	3.63	
3	7	10	2.0	0.06	0.4250	21.43	4.28	
3	7	10	2.0	0.06	0.4396	23.55	4.71	
3	8	10	0	0.6	0.3849	15.62	3.12	3.84
3	8	10	0	0.6	0.4021	18.11	3.62	
3	8	10	0	0.6	0.4489	24.89	4.97	
3	8	10	0	0.6	0.4033	18.28	3.65	
3	9	10	0.5	0.6	0.4464	24.53	4.90	4.41
3	9	10	0.5	0.6	0.4014	18.01	3.60	
3	9	10	0.5	0.6	0.4405	23.68	4.73	
3	9	10	0.5	0.6				
3	10	10	2.0	0.6	0.4528	25.46	5.09	6.22
3	10	10	2.0	0.6	0.4990	32.15	6.43	
3	10	10	2.0	0.6	0.4912	31.02	6.20	
3	10	10	2.0	0.6	0.5246	35.86	7.17	
4	1	0	0	0	0.3857	55.09	11.01	11.34
4	1	0	0	0	0.4155	59.67	11.93	
4	1	0	0	0	0.3912	55.94	11.18	
4	1	0	0	0	0.3934	56.27	11.25	
4	2	10	0	0	0.3807	54.32	10.86	11.37
4	2	10	0	0	0.4033	57.80	11.56	
4	2	10	0	0	0.4021	57.61	11.52	
4	2	10	0	0	0.4024	57.66	11.53	
4	3	10	0.5	0	0.4182	60.09	12.01	11.60
4	3	10	0.5	0	0.3906	55.84	11.16	
4	3	10	0.5	0	0.4086	58.61	11.72	
4	3	10	0.5	0	0.4019	57.58	11.51	
4	4	10	2.0	0	0.4116	59.07	11.81	12.26
4	4	10	2.0	0	0.4111	59.00	11.80	

4	4	10	2.0	0	0.4245	61.06	12.21	
4	4	10	2.0	0	0.4572	66.09	13.21	
4	5	10	0	0.06	0.4026	57.69	11.53	11.58
4	5	10	0	0.06	0.4147	59.55	11.91	
4	5	10	0	0.06	0.3945	56.44	11.28	
4	5	10	0	0.06	0.4041	57.92	11.58	
4	6	10	0.5	0.06	0.3851	55.00	11.00	11.46
4	6	10	0.5	0.06	0.4333	62.41	12.48	
4	6	10	0.5	0.06	0.3831	54.69	10.93	
4	6	10	0.5	0.06	0.3992	57.17	11.43	
4	7	10	2.0	0.06	0.3919	56.04	11.20	12.19
4	7	10	2.0	0.06	0.4294	61.81	12.36	
4	7	10	2.0	0.06	0.4261	61.30	12.26	
4	7	10	2.0	0.06	0.4481	64.69	12.93	
4	8	10	0	0.6	0.4147	59.55	11.91	11.64
4	8	10	0	0.6	0.4117	59.09	11.81	
4	8	10	0	0.6	0.3917	56.01	11.20	
4	8	10	0	0.6				
4	9	10	0.5	0.6	0.4149	59.58	11.91	12.50
4	9	10	0.5	0.6	0.4353	62.72	12.54	
4	9	10	0.5	0.6	0.4528	65.41	13.08	
4	9	10	0.5	0.6	0.4326	62.30	12.46	
4	10	10	2.0	0.6	0.4058	58.18	11.63	11.51
4	10	10	2.0	0.6	0.3973	56.87	11.37	
4	10	10	2.0	0.6	0.4025	57.67	11.53	
4	10	10	2.0	0.6	0.4020	57.60	11.52	
5	1	0	0	0	0.4544	65.66	13.13	13.17
5	1	0	0	0	0.4744	68.74	13.74	
5	1	0	0	0	0.4410	63.60	12.72	
5	1	0	0	0	0.4529	65.43	13.08	
5	2	10	0	0	0.4289	61.74	12.34	12.66
5	2	10	0	0	0.4460	64.37	12.87	
5	2	10	0	0	0.4504	65.04	13.00	
5	2	10	0	0	0.4319	62.20	12.44	
5	3	10	0.5	0	0.4406	63.54	12.70	14.36
5	3	10	0.5	0				
5	3	10	0.5	0	0.4303	61.95	12.39	
5	3	10	0.5	0	0.6122	89.94	17.98	
5	4	10	2.0	0	0.4232	60.86	12.17	14.80
5	4	10	2.0	0	0.4201	60.38	12.07	
5	4	10	2.0	0	0.4482	64.70	12.94	
5	4	10	2.0	0	0.7438	110.18	22.03	
5	5	10	0	0.06	0.4182	60.09	12.01	12.51
5	5	10	0	0.06	0.4303	61.95	12.39	
5	5	10	0	0.06	0.4248	61.10	12.22	
5	5	10	0	0.06	0.4634	67.04	13.04	

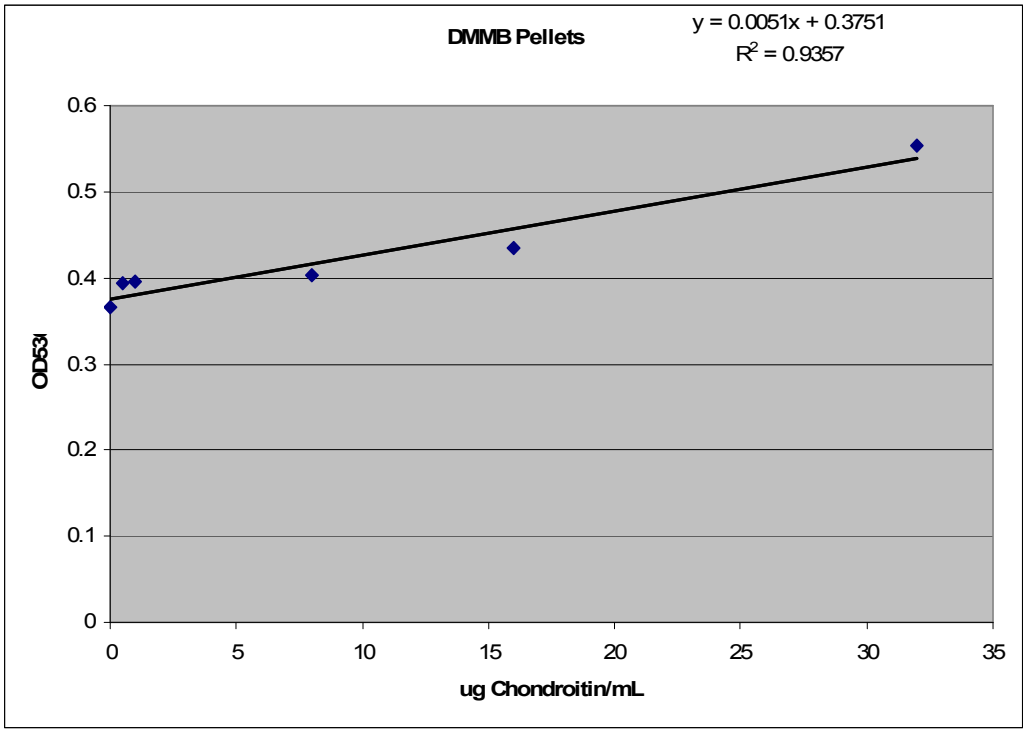
5	6	10	0.5	0.06	0.4554	65.81	13.16	12.59
5	6	10	0.5	0.06	0.4301	61.92	12.38	
5	6	10	0.5	0.06	0.4264	61.35	12.27	
5	6	10	0.5	0.06	0.4359	62.81	12.56	
5	7	10	2.0	0.06				12.69
5	7	10	2.0	0.06				
5	7	10	2.0	0.06	0.4401	63.46	12.69	
5	7	10	2.0	0.06				
5	8	10	0	0.6	0.4301	61.92	12.38	13.60
5	8	10	0	0.6	0.4470	64.52	12.90	
5	8	10	0	0.6	0.5572	81.47	16.29	
5	8	10	0	0.6	0.4453	64.26	12.85	
5	9	10	0.5	0.6	0.4386	63.23	12.64	13.83
5	9	10	0.5	0.6	0.4493	64.87	12.97	
5	9	10	0.5	0.6	0.4528	65.41	13.08	
5	9	10	0.5	0.6	0.5682	83.17	16.63	
5	10	10	2.0	0.6	0.6144	90.27	18.05	18.29
5	10	10	2.0	0.6	0.6477	95.40	19.08	
5	10	10	2.0	0.6				
5	10	10	2.0	0.6	0.6045	88.75	17.75	
6	1	0	0	0	0.3827	17.71	3.54	3.63
6	1	0	0	0	0.3960	19.72	3.94	
6	1	0	0	0	0.3816	17.54	3.50	
6	1	0	0	0	0.3824	17.66	3.53	
6	2	10	0	0	0.3608	14.39	2.87	3.17
6	2	10	0	0	0.3765	16.77	3.35	
6	2	10	0	0	0.3708	15.90	3.18	
6	2	10	0	0	0.3741	16.40	3.28	
6	3	10	0.5	0	0.3903	18.86	3.77	2.95
6	3	10	0.5	0	0.3536	13.15	2.63	
6	3	10	0.5	0	0.3552	13.54	2.70	
6	3	10	0.5	0	0.3546	13.45	2.69	
6	4	10	2.0	0	0.3456	12.09	2.41	2.89
6	4	10	2.0	0	0.3545	13.43	2.68	
6	4	10	2.0	0	0.3672	15.36	3.07	
6	4	10	2.0	0	0.3784	17.06	3.41	
6	5	10	0	0.06	0.3785	17.07	3.41	3.28
6	5	10	0	0.06	0.3814	17.51	3.50	
6	5	10	0	0.06	0.3551	13.53	2.70	
6	5	10	0	0.06	0.3815	17.53	3.50	
6	6	10	0.5	0.06	0.4015	20.56	4.11	3.95
6	6	10	0.5	0.06	0.4002	20.36	4.07	
6	6	10	0.5	0.06	0.3989	20.16	4.03	
6	6	10	0.5	0.06	0.3850	18.06	3.61	
6	7	10	2.0	0.06	0.3503	12.80	2.56	3.23
6	7	10	2.0	0.06	0.3947	19.53	3.90	

6	7	10	2.0	0.06	0.3697	15.74	3.14	
6	7	10	2.0	0.06	0.3759	16.68	3.33	
6	8	10	0	0.6	0.3505	12.83	2.56	2.83
6	8	10	0	0.6	0.3724	16.15	3.23	
6	8	10	0	0.6	0.3421	11.56	2.31	
6	8	10	0	0.6	0.3725	16.16	3.23	
6	9	10	0.5	0.6	0.3871	18.37	3.67	3.35
6	9	10	0.5	0.6	0.3874	18.42	3.68	
6	9	10	0.5	0.6	0.3461	12.16	2.43	
6	9	10	0.5	0.6	0.3852	18.09	3.61	
6	10	10	2.0	0.6	0.3908	18.93	3.78	3.94
6	10	10	2.0	0.6	0.4075	21.46	4.29	
6	10	10	2.0	0.6	0.3997	20.28	4.05	
6	10	10	2.0	0.6	0.3856	18.15	3.63	
7	1	0	0	0	0.3401	48.07	9.61	10.49
7	1	0	0	0	0.3704	52.74	10.54	
7	1	0	0	0	0.3658	52.03	10.40	
7	1	0	0	0	0.3982	57.01	11.40	
7	2	10	0	0	0.3833	54.72	10.94	10.62
7	2	10	0	0	0.3816	54.46	10.89	
7	2	10	0	0	0.3592	51.01	10.20	
7	2	10	0	0	0.3669	52.20	10.44	
7	3	10	0.5	0	0.3985	57.06	11.41	10.76
7	3	10	0.5	0	0.3960	56.67	11.33	
7	3	10	0.5	0	0.3471	49.15	9.83	
7	3	10	0.5	0	0.3681	52.38	10.47	
7	4	10	2.0	0	0.3790	54.06	10.81	10.37
7	4	10	2.0	0	0.3602	51.17	10.23	
7	4	10	2.0	0	0.3639	51.74	10.34	
7	4	10	2.0	0	0.3562	50.55	10.11	
7	5	10	0	0.06	0.3459	48.97	9.79	9.83
7	5	10	0	0.06	0.3516	49.84	9.96	
7	5	10	0	0.06	0.3717	52.94	10.58	
7	5	10	0	0.06	0.3192	44.86	8.97	
7	6	10	0.5	0.06	0.3594	51.04	10.20	10.26
7	6	10	0.5	0.06	0.3794	53.43	10.68	
7	6	10	0.5	0.06	0.3636	51.69	10.33	
7	6	10	0.5	0.06	0.3465	49.06	9.81	
7	7	10	2.0	0.06	0.3846	54.92	10.98	12.02
7	7	10	2.0	0.06	0.3998	57.26	11.45	
7	7	10	2.0	0.06	0.4448	64.18	12.83	
7	7	10	2.0	0.06	0.4444	64.12	12.82	
7	8	10	0	0.6	0.4011	57.46	11.49	10.59
7	8	10	0	0.6	0.3751	53.46	10.69	
7	8	10	0	0.6	0.3751	53.46	10.69	
7	8	10	0	0.6	0.3359	47.43	9.48	

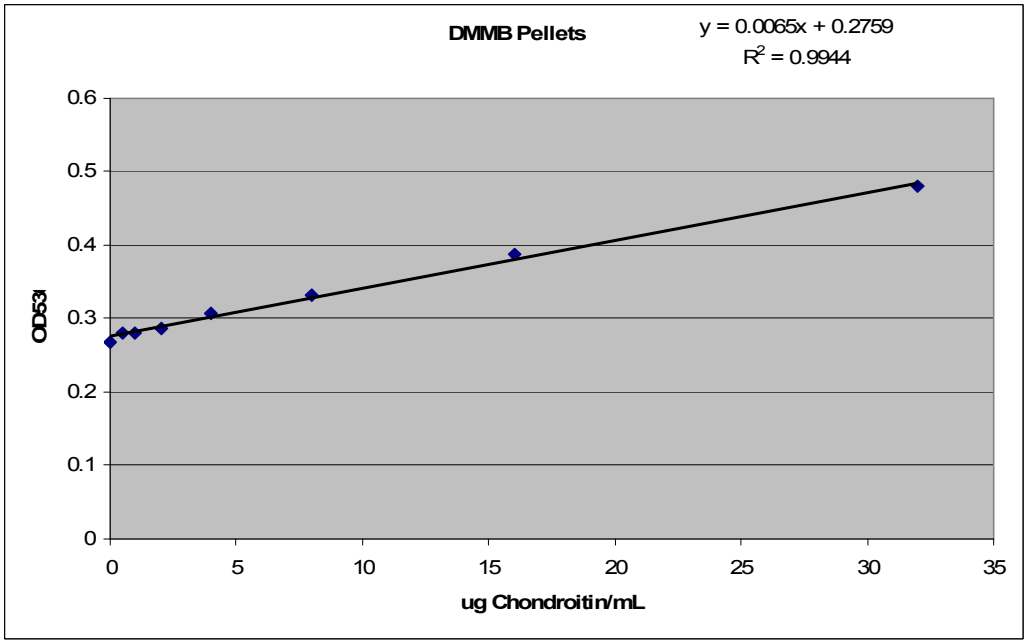
7	9	10	0.5	0.6	0.3911	55.92	11.18	11.44
7	9	10	0.5	0.6	0.4301	61.92	12.38	
7	9	10	0.5	0.6	0.4224	60.74	12.14	
7	9	10	0.5	0.6	0.3541	50.23	10.04	
7	10	10	2.0	0.6	0.4118	59.10	11.82	11.98
7	10	10	2.0	0.6	0.4342	62.55	12.51	
7	10	10	2.0	0.6	0.3950	56.52	11.30	
7	10	10	2.0	0.6	0.4268	61.41	12.28	

Standard curves for DMMB analysis (pellet):

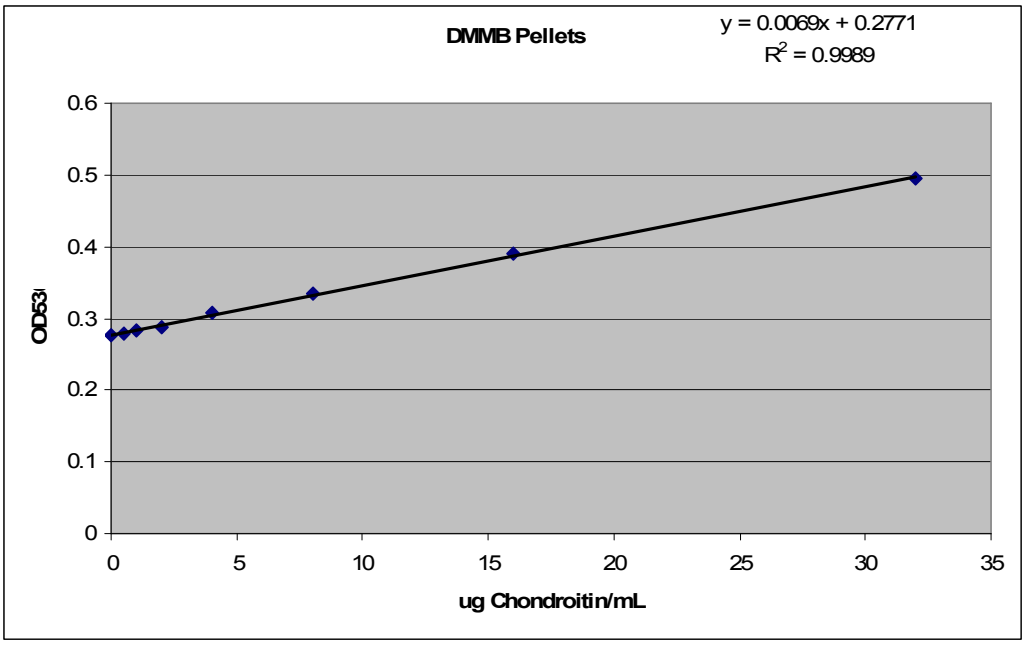
Horse 1



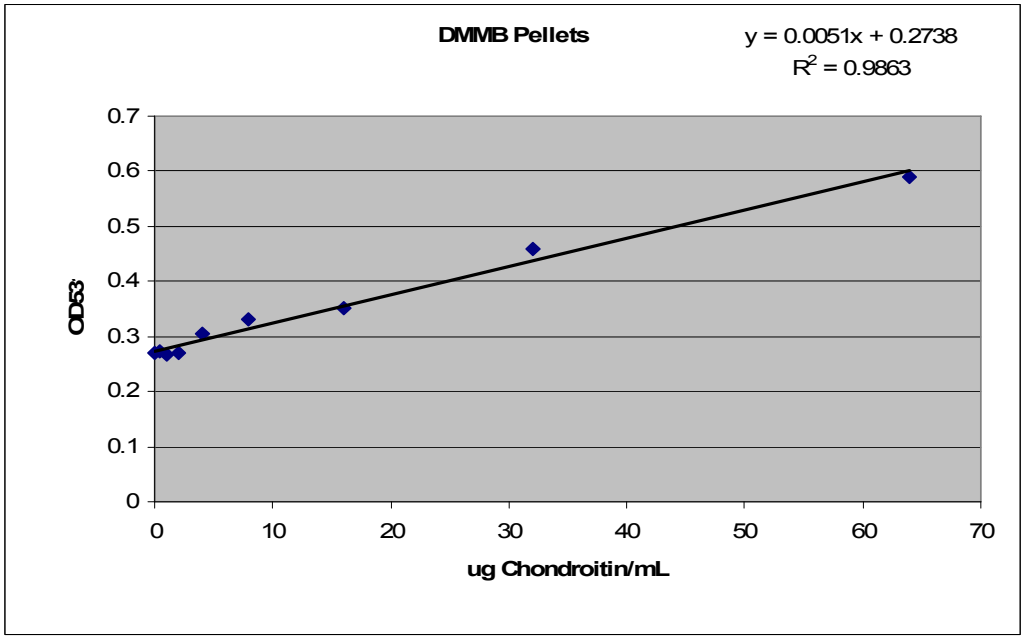
Horse 2



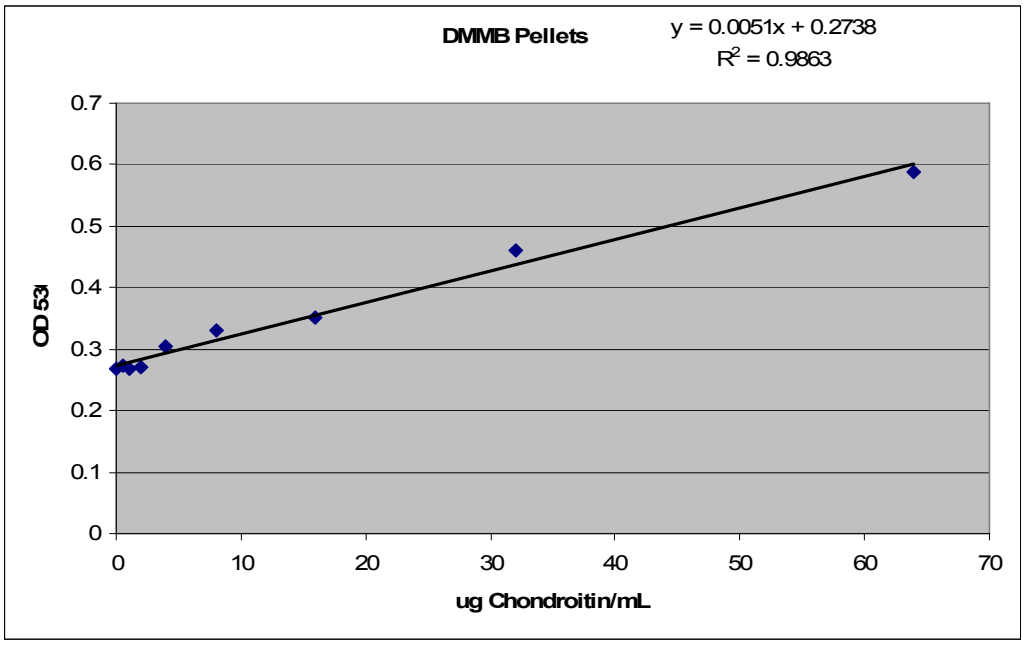
Horse 3



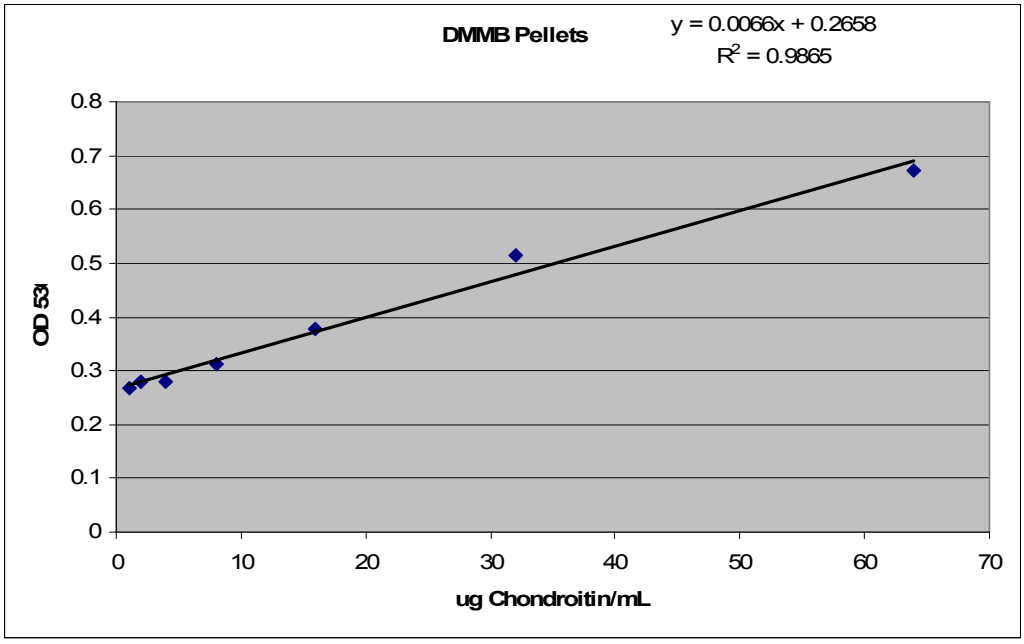
Horse 4



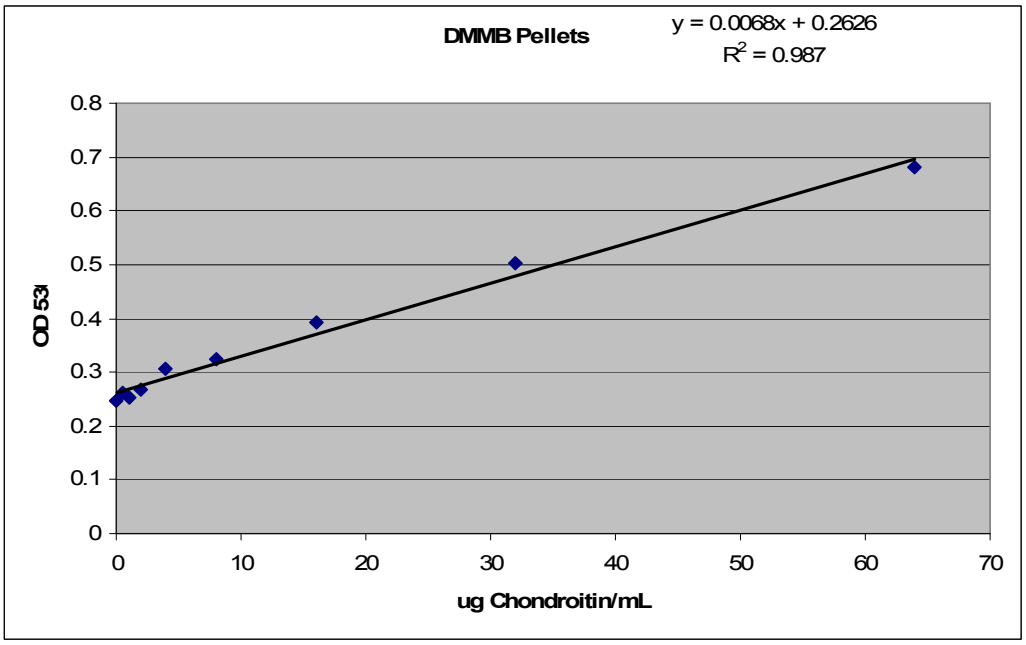
Horse 5



Horse 6



Horse 7


DMMB Pellets
y = 0.0068x + 0.2626
R2 = 0.987
OD 531
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
70
ug Chondroitin/mL

APPENDIX D. DMMB MEDIA DATA

Horse	Tx Group	IL-1 ug/mL	HA mg/mL	TA mg/mL	Optical Density	Chondr. ug/mL	Total/ Pellet	Avg per tx group
1	1	0	0	0	0.4963	15.05	7.52	8.84
1	1	0	0	0	0.5174	18.75	9.37	
1	1	0	0	0				
1	1	0	0	0	0.5202	19.24	9.62	
1	2	10	0	0	0.5232	19.77	9.88	11.06
1	2	10	0	0	0.5629	26.73	13.36	
1	2	10	0	0	0.5291	20.80	10.40	
1	2	10	0	0	0.5313	21.19	10.59	
1	3	10	0.5	0				
1	3	10	0.5	0				
1	3	10	0.5	0				
1	3	10	0.5	0				
1	4	10	2.0	0	0.5495	24.38	12.19	11.28
1	4	10	2.0	0	0.5369	22.17	11.08	
1	4	10	2.0	0	0.5283	20.66	10.33	
1	4	10	2.0	0	0.5420	23.07	11.53	
1	5	10	0	0.06	0.6027	33.71	16.85	14.50
1	5	10	0	0.06	0.5566	25.63	12.81	
1	5	10	0	0.06	0.5432	23.28	11.64	
1	5	10	0	0.06	0.6010	33.42	16.71	
1	6	10	0.5	0.06				
1	6	10	0.5	0.06				
1	6	10	0.5	0.06				
1	6	10	0.5	0.06				
1	7	10	2.0	0.06	0.5774	29.28	14.64	15.35
1	7	10	2.0	0.06	0.5952	32.40	16.20	
1	7	10	2.0	0.06	0.5701	28.00	14.00	
1	7	10	2.0	0.06	0.5995	33.15	16.57	
1	8	10	0	0.6	0.7144	53.31	26.65	23.05
1	8	10	0	0.6	0.6896	48.96	24.48	
1	8	10	0	0.6	0.6184	36.47	18.23	
1	8	10	0	0.6	0.6711	45.71	22.85	
1	9	10	0.5	0.6				
1	9	10	0.5	0.6				
1	9	10	0.5	0.6				
1	9	10	0.5	0.6				
1	10	10	2.0	0.6	0.6758	46.54	23.27	22.38
1	10	10	2.0	0.6	0.7058	51.80	25.90	
1	10	10	2.0	0.6	0.5848	30.57	15.28	
1	10	10	2.0	0.6	0.6964	50.15	25.07	
2	1	0	0	0	0.3486	13.42	6.71	10.14
2	1	0	0	0	0.3707	17.93	8.96	

2	1	0	0	0	0.4171	27.40	13.70	
2	1	0	0	0	0.3926	22.40	11.20	
2	2	10	0	0	0.3392	11.51	5.75	10.98
2	2	10	0	0	0.3587	15.48	7.74	
2	2	10	0	0	0.4663	37.44	18.72	
2	2	10	0	0	0.3975	23.40	11.70	
2	3	10	0.5	0	0.6238	69.59	34.79	33.79
2	3	10	0.5	0	0.5782	60.28	30.14	
2	3	10	0.5	0	0.6723	79.48	39.74	
2	3	10	0.5	0	0.5816	60.97	30.48	
2	4	10	2.0	0	0.4403	32.14	16.07	17.34
2	4	10	2.0	0	0.4906	42.40	21.20	
2	4	10	2.0	0	0.4587	35.89	17.94	
2	4	10	2.0	0	0.4217	28.34	14.17	
2	5	10	0	0.06	0.4200	28.00	14.00	17.59
2	5	10	0	0.06	0.4797	40.18	20.09	
2	5	10	0	0.06	0.4611	36.38	18.19	
2	5	10	0	0.06	0.4602	36.20	18.10	
2	6	10	0.5	0.06	0.5677	58.14	29.07	30.50
2	6	10	0.5	0.06	0.5606	56.69	28.34	
2	6	10	0.5	0.06	0.6388	72.65	36.32	
2	6	10	0.5	0.06	0.5600	56.57	28.28	
2	7	10	2.0	0.06	0.5495	54.42	27.21	21.16
2	7	10	2.0	0.06	0.4665	37.48	18.74	
2	7	10	2.0	0.06	0.5091	46.18	23.09	
2	7	10	2.0	0.06	0.4359	31.24	15.62	
2	8	10	0	0.6	0.5372	51.91	25.95	27.14
2	8	10	0	0.6	0.5686	58.32	29.16	
2	8	10	0	0.6	0.5411	52.71	26.35	
2	8	10	0	0.6	0.5482	54.16	27.08	
2	9	10	0.5	0.6	0.5278	50.00	25.00	25.86
2	9	10	0.5	0.6	0.5709	58.79	29.39	
2	9	10	0.5	0.6	0.5268	49.79	24.89	
2	9	10	0.5	0.6	0.5197	48.34	24.17	
2	10	10	2.0	0.6	0.5166	47.71	23.85	29.05
2	10	10	2.0	0.6	0.5554	55.63	27.81	
2	10	10	2.0	0.6	0.6137	67.53	33.76	
2	10	10	2.0	0.6	0.5845	61.57	30.78	
3	1	0	0	0	0.4390	23.79	11.89	14.78
3	1	0	0	0	0.4652	27.70	13.85	
3	1	0	0	0	0.4919	31.68	15.84	
3	1	0	0	0	0.5150	35.13	17.56	
3	2	10	0	0	0.4647	27.62	13.81	14.37
3	2	10	0	0	0.5415	39.08	19.54	
3	2	10	0	0	0.4293	22.34	11.17	
3	2	10	0	0	0.4534	25.94	12.97	

3	3	10	0.5	0	0.4793	29.80	14.90	21.62
3	3	10	0.5	0	0.4893	31.29	15.64	
3	3	10	0.5	0	0.8275	81.77	40.88	
3	3	10	0.5	0	0.4813	30.10	15.05	
3	4	10	2.0	0	0.5285	37.14	18.57	23.10
3	4	10	2.0	0	0.7283	66.97	33.48	
3	4	10	2.0	0	0.5257	36.73	18.36	
3	4	10	2.0	0	0.5745	44.01	22.00	
3	5	10	0	0.06	0.5468	39.88	19.94	21.17
3	5	10	0	0.06	0.5650	42.59	21.29	
3	5	10	0	0.06	0.5694	43.25	21.62	
3	5	10	0	0.06	0.5722	43.67	21.83	
3	6	10	0.5	0.06	0.5725	43.71	21.85	21.93
3	6	10	0.5	0.06	0.5628	42.26	21.13	
3	6	10	0.5	0.06	0.6256	51.64	25.82	
3	6	10	0.5	0.06	0.5333	37.86	18.93	
3	7	10	2.0	0.06	0.5714	43.55	21.77	27.26
3	7	10	2.0	0.06	0.6002	47.85	23.92	
3	7	10	2.0	0.06	0.7850	75.43	37.71	
3	7	10	2.0	0.06	0.6233	51.29	25.64	
3	8	10	0	0.6	0.6535	55.80	27.90	28.84
3	8	10	0	0.6	0.6890	61.10	30.55	
3	8	10	0	0.6	0.7147	64.94	32.47	
3	8	10	0	0.6	0.6071	48.88	24.44	
3	9	10	0.5	0.6	0.6251	51.56	25.78	26.74
3	9	10	0.5	0.6	0.6303	52.34	26.17	
3	9	10	0.5	0.6	0.6448	54.50	27.25	
3	9	10	0.5	0.6	0.6519	55.56	27.78	
3	10	10	2.0	0.6	0.6028	48.23	24.11	26.06
3	10	10	2.0	0.6	0.6343	52.94	26.47	
3	10	10	2.0	0.6	0.6252	51.58	25.79	
3	10	10	2.0	0.6	0.6534	55.79	27.89	
4	1	0	0	0	0.5060	41.88	20.94	19.65
4	1	0	0	0	0.4562	33.72	16.86	
4	1	0	0	0	0.4412	31.26	15.63	
4	1	0	0	0	0.5579	50.39	25.19	
4	2	10	0	0	0.4818	37.91	18.95	17.63
4	2	10	0	0	0.4350	30.24	15.12	
4	2	10	0	0	0.4098	26.11	13.05	
4	2	10	0	0	0.5358	46.77	23.38	
4	3	10	0.5	0	0.6852	71.26	35.63	37.40
4	3	10	0.5	0	0.6108	59.06	29.53	
4	3	10	0.5	0	0.7785	86.55	43.27	
4	3	10	0.5	0	0.7529	82.36	41.18	
4	4	10	2.0	0	0.4794	37.52	18.76	21.83
4	4	10	2.0	0	0.5464	48.50	24.25	

4	4	10	2.0	0	0.5675	51.96	25.98	
4	4	10	2.0	0	0.4743	36.68	18.34	
4	5	10	0	0.06	0.4563	33.73	16.86	20.64
4	5	10	0	0.06	0.5791	53.86	26.93	
4	5	10	0	0.06	0.4580	34.01	17.00	
4	5	10	0	0.06	0.5163	43.57	21.78	
4	6	10	0.5	0.06	0.7374	79.81	39.90	39.66
4	6	10	0.5	0.06	0.7725	85.57	42.78	
4	6	10	0.5	0.06	0.7450	81.06	40.53	
4	6	10	0.5	0.06	0.6827	70.85	35.42	
4	7	10	2.0	0.06	0.5888	55.45	27.72	27.86
4	7	10	2.0	0.06	0.6272	61.75	30.87	
4	7	10	2.0	0.06	0.5637	51.34	25.67	
4	7	10	2.0	0.06	0.5822	54.37	27.18	
4	8	10	0	0.6	0.7105	75.40	37.70	35.28
4	8	10	0	0.6	0.6497	65.44	32.72	
4	8	10	0	0.6	0.7156	76.24	38.12	
4	8	10	0	0.6	0.6482	65.19	32.59	
4	9	10	0.5	0.6	0.7328	79.06	39.53	34.20
4	9	10	0.5	0.6	0.6238	61.19	30.59	
4	9	10	0.5	0.6	0.5908	55.78	27.89	
4	9	10	0.5	0.6	0.7239	77.60	38.80	
4	10	10	2.0	0.6	0.5470	48.60	24.30	29.31
4	10	10	2.0	0.6	0.6568	66.60	33.30	
4	10	10	2.0	0.6	0.6516	65.75	32.87	
4	10	10	2.0	0.6	0.5770	53.52	26.76	
5	1	0	0	0	0.4759	36.95	18.47	18.22
5	1	0	0	0	0.4745	36.72	18.36	
5	1	0	0	0	0.4654	35.22	17.61	
5	1	0	0	0	0.4757	36.91	18.45	
5	2	10	0	0	0.4230	28.27	14.13	13.48
5	2	10	0	0	0.4648	35.13	17.56	
5	2	10	0	0	0.3878	22.50	11.25	
5	2	10	0	0	0.3847	22.00	11.00	
5	3	10	0.5	0	0.9184	109.49	54.74	48.77
5	3	10	0.5	0	0.8348	95.78	47.89	
5	3	10	0.5	0	0.8097	91.67	45.83	
5	3	10	0.5	0	0.8191	93.21	46.60	
5	4	10	2.0	0	0.6281	61.90	30.95	27.69
5	4	10	2.0	0	0.5190	44.01	22.00	
5	4	10	2.0	0	0.5481	48.78	24.39	
5	4	10	2.0	0	0.6581	66.81	33.40	
5	5	10	0	0.06	0.4686	35.75	17.87	21.27
5	5	10	0	0.06	0.5985	57.04	28.52	
5	5	10	0	0.06	0.5421	47.80	23.90	
5	5	10	0	0.06	0.4311	29.60	14.80	

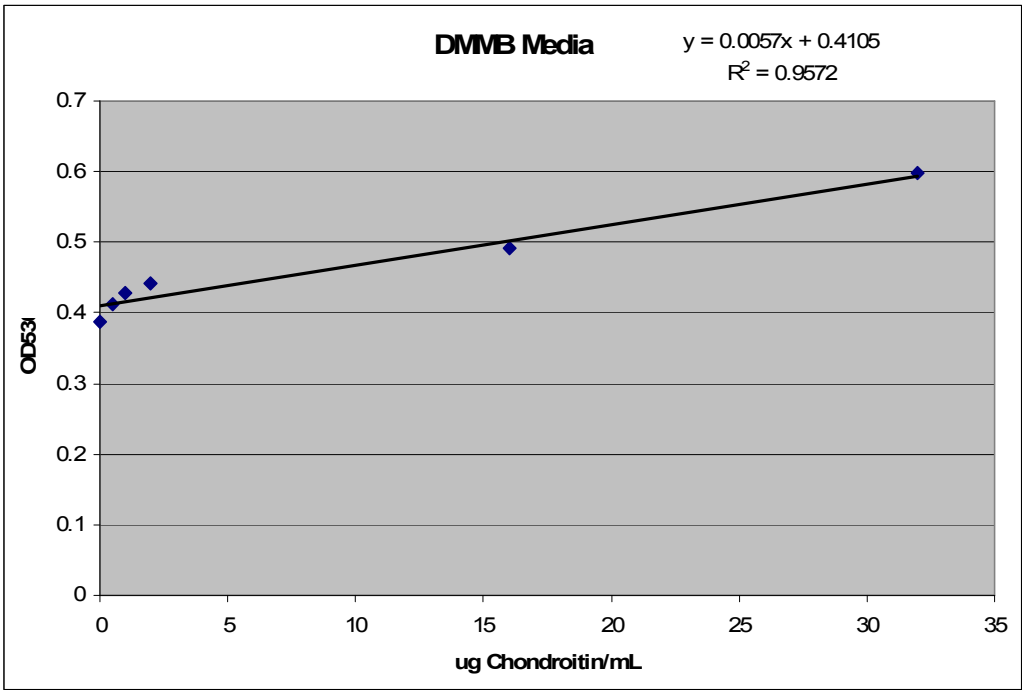
5	6	10	0.5	0.06	0.8370	96.14	48.07	43.13
5	6	10	0.5	0.06	0.7693	85.04	42.52	
5	6	10	0.5	0.06	0.7144	76.04	38.02	
5	6	10	0.5	0.06	0.7861	87.80	43.90	
5	7	10	2.0	0.06	0.5909	55.80	27.90	29.13
5	7	10	2.0	0.06	0.5553	49.96	24.98	
5	7	10	2.0	0.06	0.6535	66.06	33.03	
5	7	10	2.0	0.06	0.6240	61.22	30.61	
5	8	10	0	0.6	0.5490	48.93	24.46	27.17
5	8	10	0	0.6	0.6340	62.86	31.43	
5	8	10	0	0.6	0.6535	66.06	33.03	
5	8	10	0	0.6	0.5890	55.49	27.74	
5	9	10	0.5	0.6	0.5962	56.67	28.33	27.15
5	9	10	0.5	0.6	0.5972	56.83	28.41	
5	9	10	0.5	0.6	0.5849	54.81	27.40	
5	9	10	0.5	0.6	0.5489	48.91	24.45	
5	10	10	2.0	0.6	0.5849	54.81	27.40	30.14
5	10	10	2.0	0.6	0.7239	77.60	38.80	
5	10	10	2.0	0.6	0.6313	62.42	31.21	
5	10	10	2.0	0.6	0.5328	46.27	23.13	
6	1	0	0	0	0.3979	20.01	10.00	10.34
6	1	0	0	0	0.4194	23.27	11.63	
6	1	0	0	0	0.3967	19.83	9.91	
6	1	0	0	0	0.3956	19.66	9.83	
6	2	10	0	0	0.3576	13.90	6.95	7.60
6	2	10	0	0	0.3615	14.50	7.25	
6	2	10	0	0	0.3710	15.93	7.96	
6	2	10	0	0	0.3749	16.53	8.26	
6	3	10	0.5	0	0.7418	72.12	36.06	37.27
6	3	10	0.5	0	0.7853	78.71	39.35	
6	3	10	0.5	0	0.7591	74.74	37.37	
6	3	10	0.5	0	0.7450	72.60	36.30	
6	4	10	2.0	0	0.5272	39.60	19.80	18.57
6	4	10	2.0	0	0.4756	31.78	15.89	
6	4	10	2.0	0	0.5152	37.78	18.89	
6	4	10	2.0	0	0.5257	39.37	19.68	
6	5	10	0	0.06	0.4451	27.16	13.58	14.71
6	5	10	0	0.06	0.4681	30.65	15.32	
6	5	10	0	0.06	0.4646	30.12	15.06	
6	5	10	0	0.06	0.4626	29.81	14.90	
6	6	10	0.5	0.06	0.6400	56.69	28.34	29.42
6	6	10	0.5	0.06	0.6656	60.57	30.28	
6	6	10	0.5	0.06	0.6442	57.33	28.66	
6	6	10	0.5	0.06	0.6672	60.81	30.40	
6	7	10	2.0	0.06	0.5901	49.13	24.56	21.14
6	7	10	2.0	0.06	0.5296	39.96	19.98	

6	7	10	2.0	0.06	0.5273	39.62	19.81	
6	7	10	2.0	0.06	0.5325	40.40	20.20	
6	8	10	0	0.6	0.5788	47.42	23.71	22.50
6	8	10	0	0.6	0.5531	43.53	21.76	
6	8	10	0	0.6	0.5621	44.89	22.44	
6	8	10	0	0.6	0.5575	44.19	22.09	
6	9	10	0.5	0.6	0.5591	44.43	22.21	24.05
6	9	10	0.5	0.6	0.6014	50.84	25.42	
6	9	10	0.5	0.6	0.6057	51.50	25.75	
6	9	10	0.5	0.6	0.5669	45.62	22.81	
6	10	10	2.0	0.6	0.6244	54.33	27.16	27.52
6	10	10	2.0	0.6	0.6744	61.90	30.95	
6	10	10	2.0	0.6	0.6198	53.63	26.81	
6	10	10	2.0	0.6	0.5980	50.33	25.16	
7	1	0	0	0	0.5054	35.70	17.85	13.45
7	1	0	0	0	0.4754	31.29	15.64	
7	1	0	0	0	0.4167	22.66	11.33	
7	1	0	0	0	0.3851	18.01	9.00	
7	2	10	0	0	0.5110	36.52	18.26	14.57
7	2	10	0	0	0.4544	28.20	14.10	
7	2	10	0	0	0.4268	24.14	12.07	
7	2	10	0	0	0.4509	27.69	13.84	
7	3	10	0.5	0	0.5498	42.23	21.11	20.75
7	3	10	0.5	0	0.5637	44.27	22.13	
7	3	10	0.5	0	0.5779	46.36	23.18	
7	3	10	0.5	0	0.4883	33.19	16.59	
7	4	10	2.0	0	0.4514	27.76	13.88	13.12
7	4	10	2.0	0	0.4292	24.50	12.25	
7	4	10	2.0	0	0.4375	25.72	12.86	
7	4	10	2.0	0	0.4465	27.04	13.52	
7	5	10	0	0.06	0.4571	28.60	14.30	14.59
7	5	10	0	0.06	0.4410	26.23	13.11	
7	5	10	0	0.06	0.4710	30.64	15.32	
7	5	10	0	0.06	0.4753	31.27	15.63	
7	6	10	0.5	0.06	0.5854	47.47	23.73	23.91
7	6	10	0.5	0.06	0.6149	51.80	25.90	
7	6	10	0.5	0.06	0.5858	47.52	23.76	
7	6	10	0.5	0.06	0.5651	44.48	22.24	
7	7	10	2.0	0.06	0.5530	42.70	21.35	18.65
7	7	10	2.0	0.06	0.4508	27.67	13.83	
7	7	10	2.0	0.06	0.5449	41.51	20.75	
7	7	10	2.0	0.06	0.5166	37.35	18.67	
7	8	10	0	0.6	0.6579	58.13	29.06	27.62
7	8	10	0	0.6	0.6692	59.79	29.89	
7	8	10	0	0.6	0.6226	52.94	26.47	
7	8	10	0	0.6	0.6033	50.10	25.05	

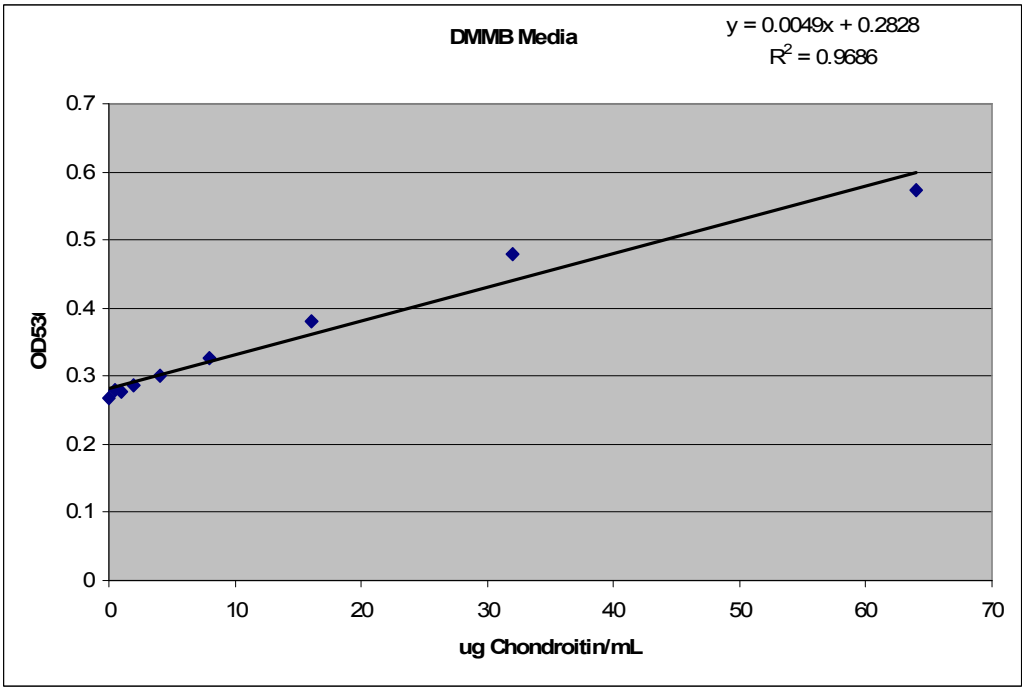
7	9	10	0.5	0.6	0.6469	56.51	28.25	26.96
7	9	10	0.5	0.6	0.6180	52.26	26.13	
7	9	10	0.5	0.6	0.6279	53.72	26.86	
7	9	10	0.5	0.6	0.6247	53.25	26.62	
7	10	10	2.0	0.6	0.7150	66.52	33.26	34.93
7	10	10	2.0	0.6	0.8209	82.10	41.5	
7	10	10	2.0	0.6	0.7420	70.50	35.25	
7	10	10	2.0	0.6	0.6728	60.32	30.16	

Standard curves for DMMB analysis (media):

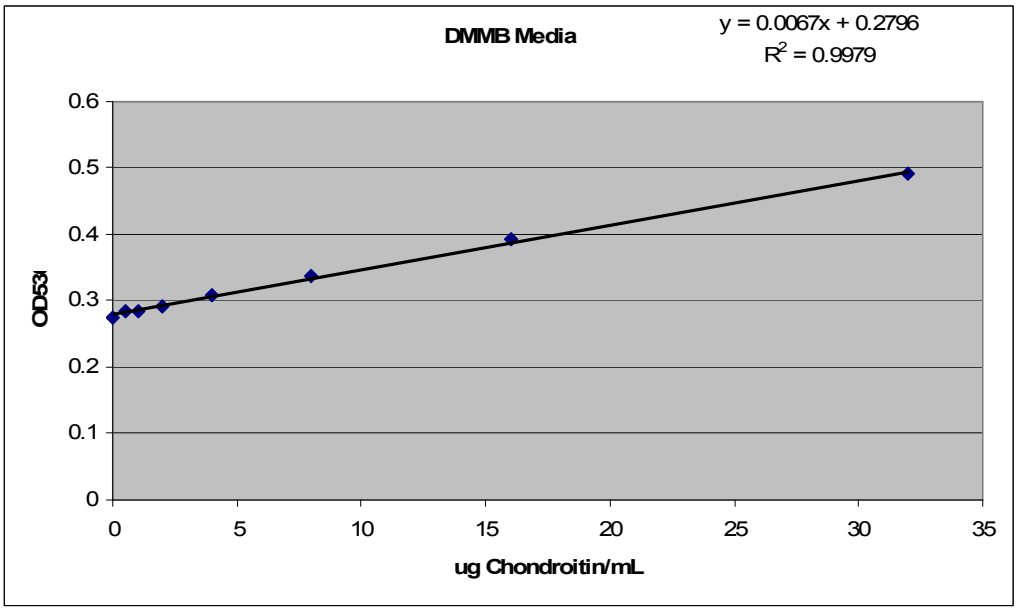
Horse 1



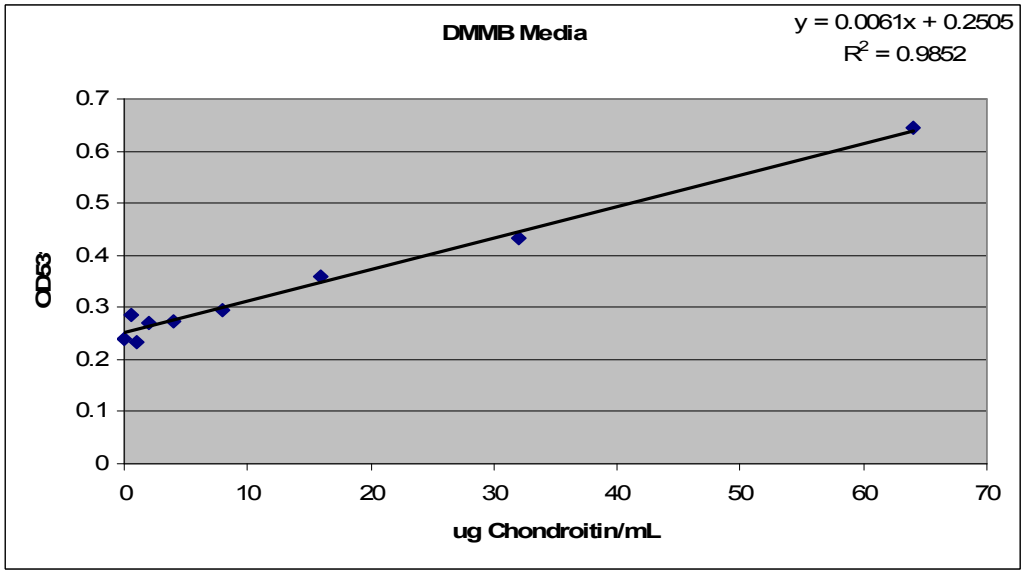
Horse 2



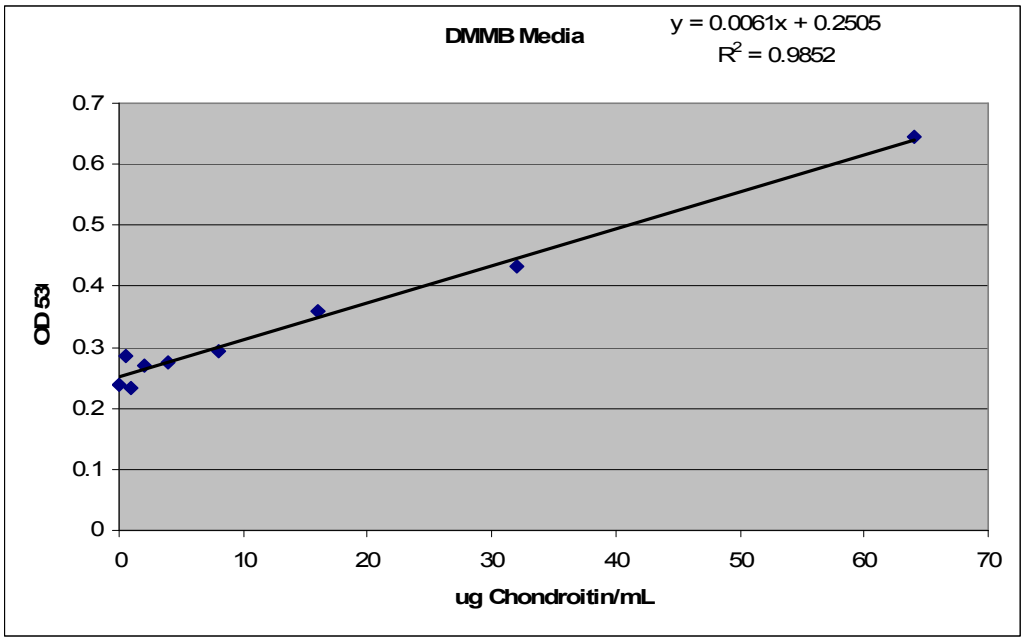
Horse 3



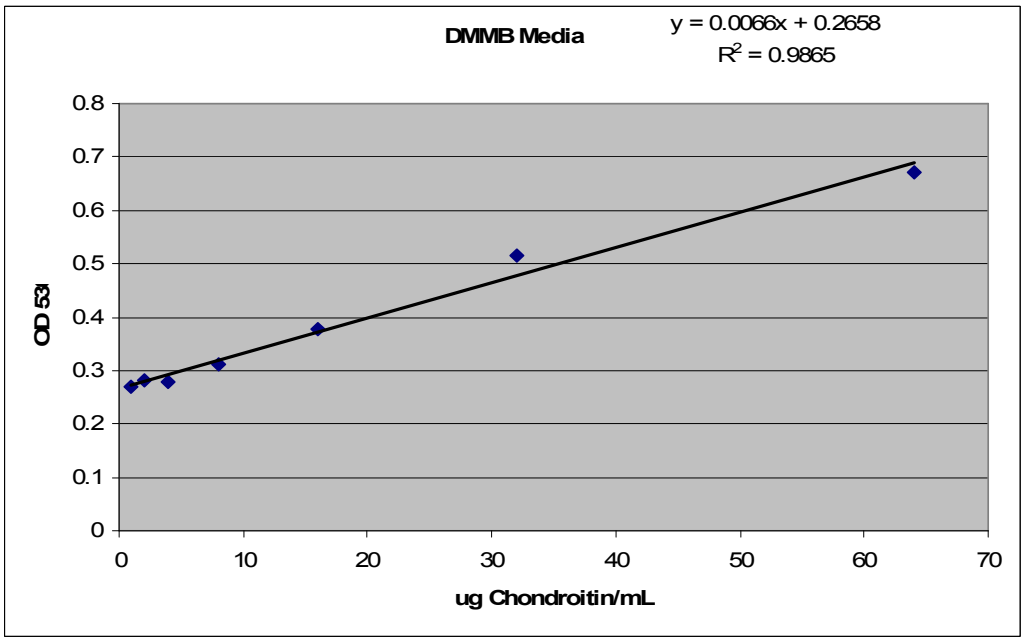
Horse 4



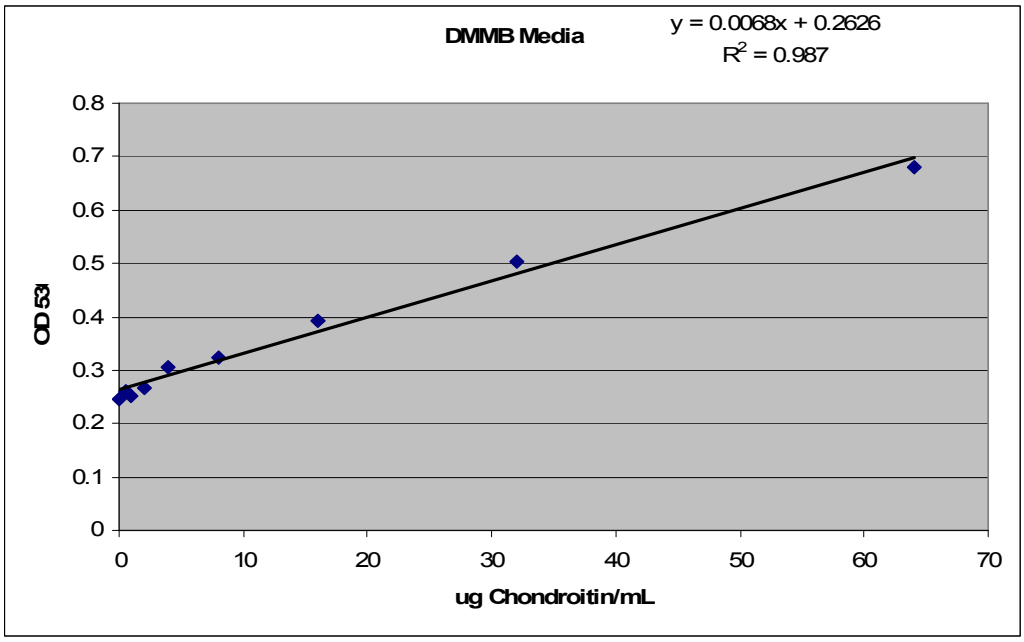
Horse 5



Horse 6



Horse 7


DMMB Media
y = 0.0068x + 0.2626
R2 = 0.987
OD 531
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
70
ug Chondroitin/mL

APPENDIX E. DNA DATA

Horse	Tx Group	IL-1 ug/mL	HA mg/mL	TA mg/mL	Fluoro units/s	DNA ug/mL	Total/ Pellet	Avg per tx group
1	1	0	0	0	45947	32.85	3.28	3.74
1	1	0	0	0	50400	36.83	3.68	
1	1	0	0	0	51759	38.05	3.80	
1	1	0	0	0	43572	30.73	3.07	
1	1	0	0	0	50476	36.90	3.69	
1	1	0	0	0	52429	38.64	3.86	
1	1	0	0	0	57907	43.54	4.35	
1	1	0	0	0	56630	42.40	4.24	
1	2	10	0	0	40349	27.85	2.78	2.46
1	2	10	0	0	37231	25.06	2.50	
1	2	10	0	0	36697	24.59	2.45	
1	2	10	0	0	34326	22.47	2.24	
1	2	10	0	0	35591	23.60	2.36	
1	2	10	0	0	26962	15.89	1.58	
1	2	10	0	0	44884	31.90	3.19	
1	2	10	0	0	37932	25.69	2.56	
1	3	10	0.5	0				
1	3	10	0.5	0				
1	3	10	0.5	0				
1	3	10	0.5	0				
1	3	10	0.5	0				
1	3	10	0.5	0				
1	3	10	0.5	0				
1	3	10	0.5	0				
1	4	10	2.0	0	50110	36.57	3.65	3.54
1	4	10	2.0	0	55799	41.66	4.16	
1	4	10	2.0	0	52743	38.93	3.89	
1	4	10	2.0	0	54009	40.06	4.00	
1	4	10	2.0	0	34538	22.66	2.26	
1	4	10	2.0	0	42701	29.95	2.99	
1	4	10	2.0	0	49942	36.42	3.64	
1	4	10	2.0	0	51053	37.42	3.74	
1	5	10	0	0.06	36625	24.52	2.45	3.16
1	5	10	0	0.06	42009	29.33	2.93	
1	5	10	0	0.06	41527	28.90	2.89	
1	5	10	0	0.06	54547	40.54	4.05	
1	5	10	0	0.06	43919	31.04	3.10	
1	5	10	0	0.06	47480	34.22	3.42	
1	5	10	0	0.06	41420	28.81	2.88	
1	5	10	0	0.06	48902	35.49	3.54	
1	6	10	0.5	0.06				
1	6	10	0.5	0.06				

1	6	10	0.5	0.06				
1	6	10	0.5	0.06				
1	6	10	0.5	0.06				
1	6	10	0.5	0.06				
1	6	10	0.5	0.06				
1	6	10	0.5	0.06				
1	7	10	2.0	0.06	28143	16.94	1.69	1.89
1	7	10	2.0	0.06				
1	7	10	2.0	0.06				
1	7	10	2.0	0.06				
1	7	10	2.0	0.06	39777	27.34	2.73	
1	7	10	2.0	0.06				
1	7	10	2.0	0.06				
1	7	10	2.0	0.06				
1	8	10	0	0.6	42312	29.60	2.96	2.68
1	8	10	0	0.6	42981	30.20	3.02	
1	8	10	0	0.6	34479	22.61	2.26	
1	8	10	0	0.6	39465	27.06	2.70	
1	8	10	0	0.6	30996	19.49	1.94	
1	8	10	0	0.6	29341	18.01	1.80	
1	8	10	0	0.6	44371	31.44	3.14	
1	8	10	0	0.6	50150	36.61	3.66	
1	9	10	0.5	0.6				
1	9	10	0.5	0.6				
1	9	10	0.5	0.6				
1	9	10	0.5	0.6				
1	9	10	0.5	0.6				
1	9	10	0.5	0.6				
1	9	10	0.5	0.6				
1	9	10	0.5	0.6				
1	10	10	2.0	0.6	39208	26.83	2.68	2.98
1	10	10	2.0	0.6	46875	33.68	3.36	
1	10	10	2.0	0.6	25423	14.51	1.45	
1	10	10	2.0	0.6	23122	12.46	1.24	
1	10	10	2.0	0.6	47700	34.42	3.44	
1	10	10	2.0	0.6	48793	35.40	3.54	
1	10	10	2.0	0.6	54183	40.21	4.02	
1	10	10	2.0	0.6	54942	40.89	4.08	
2	1	0	0	0				1.60
2	1	0	0	0	26974	13.50	1.35	
2	1	0	0	0	38043	22.04	2.20	
2	1	0	0	0	25996	12.74	1.27	
2	2	10	0	0	27021	13.53	1.35	1.17
2	2	10	0	0	26342	13.01	1.30	
2	2	10	0	0	24079	11.26	1.12	
2	2	10	0	0	21294	9.11	0.91	

2	3	10	0.5	0	36552	20.89	2.08	2.57
2	3	10	0.5	0	46523	28.58	2.85	
2	3	10	0.5	0	53013	33.59	3.35	
2	3	10	0.5	0	35275	19.90	1.99	
2	4	10	2.0	0	32503	17.76	1.77	1.65
2	4	10	2.0	0	29088	15.13	1.51	
2	4	10	2.0	0	22061			
2	4	10	2.0	0	31271	16.81	1.68	
2	5	10	0	0.06	29047	15.10	1.51	1.59
2	5	10	0	0.06	29461	15.42	1.54	
2	5	10	0	0.06	30644	16.33	1.63	
2	5	10	0	0.06	31494	16.99	1.69	
2	6	10	0.5	0.06	32289	17.60	1.76	1.85
2	6	10	0.5	0.06	38016	22.02	2.20	
2	6	10	0.5	0.06	28609	14.76	1.47	
2	6	10	0.5	0.06	35389	19.99	1.99	
2	7	10	2.0	0.06	44990	27.40	2.74	2.54
2	7	10	2.0	0.06	33956	18.89	1.88	
2	7	10	2.0	0.06	49305	30.73	3.07	
2	7	10	2.0	0.06	41644	24.82	2.48	
2	8	10	0	0.6				1.94
2	8	10	0	0.6	34526	19.33	1.93	
2	8	10	0	0.6	33053	18.19	1.81	
2	8	10	0	0.6	36380	20.76	2.07	
2	9	10	0.5	0.6	34706	19.46	1.94	2.16
2	9	10	0.5	0.6	41793	24.93	2.49	
2	9	10	0.5	0.6	26215	12.91	1.29	
2	9	10	0.5	0.6	47604	29.42	2.94	
2	10	10	2.0	0.6	29916	15.77	1.57	2.55
2	10	10	2.0	0.6	49609	30.97	3.09	
2	10	10	2.0	0.6	65000	42.84	4.28	
2	10	10	2.0	0.6	26065	12.80	1.28	
3	1	0	0	0	21792	9.10	0.91	0.96
3	1	0	0	0	21179	8.71	0.87	
3	1	0	0	0	23365	10.11	1.01	
3	1	0	0	0	23953	10.49	1.04	
3	2	10	0	0	23514	10.21	1.02	1.09
3	2	10	0	0	24806	11.04	1.10	
3	2	10	0	0	25654	11.59	1.15	
3	2	10	0	0	24405	10.78	1.07	
3	3	10	0.5	0	23050	9.91	0.99	0.96
3	3	10	0.5	0				
3	3	10	0.5	0	21298	8.78	0.87	
3	3	10	0.5	0	23419	10.15	1.01	
3	4	10	2.0	0				1.14
3	4	10	2.0	0	25134	11.25	1.12	

3	4	10	2.0	0	24491	10.84	1.08	
3	4	10	2.0	0	26765	12.30	1.23	
3	5	10	0	0.06	18926	7.26	0.72	0.94
3	5	10	0	0.06	24689	10.97	1.09	
3	5	10	0	0.06	20745	8.43	0.84	
3	5	10	0	0.06	24784	11.03	1.10	
3	6	10	0.5	0.06	25432	11.44	1.14	1.14
3	6	10	0.5	0.06	24932	11.12	1.11	
3	6	10	0.5	0.06	26784	12.32	1.23	
3	6	10	0.5	0.06	24276	10.70	1.07	
3	7	10	2.0	0.06	26167	11.92	1.19	1.31
3	7	10	2.0	0.06	25370	11.40	1.14	
3	7	10	2.0	0.06	27258	12.62	1.26	
3	7	10	2.0	0.06	33353	16.54	1.65	
3	8	10	0	0.6	23735	10.35	1.03	1.06
3	8	10	0	0.6	24372	10.76	1.07	
3	8	10	0	0.6	22563	9.60	0.96	
3	8	10	0	0.6	26012	11.82	1.18	
3	9	10	0.5	0.6	33997	16.96	1.69	1.19
3	9	10	0.5	0.6	22373	9.48	0.94	
3	9	10	0.5	0.6	22506	9.56	0.95	
3	9	10	0.5	0.6	25793	11.68	1.16	
3	10	10	2.0	0.6				1.49
3	10	10	2.0	0.6	37595	19.27	1.92	
3	10	10	2.0	0.6	21359	8.82	0.88	
3	10	10	2.0	0.6	33717	16.78	1.67	
4	1	0	0	0	25068	11.69	1.16	1.98
4	1	0	0	0	36713	21.55	2.15	
4	1	0	0	0	36050	20.99	1.09	
4	1	0	0	0	40910	25.10	1.51	
4	2	10	0	0	26989	13.31	1.33	1.70
4	2	10	0	0	24942	11.58	1.15	
4	2	10	0	0	35423	20.46	2.04	
4	2	10	0	0	38461	23.03	2.30	
4	3	10	0.5	0	39093	23.56	2.35	2.22
4	3	10	0.5	0	34355	19.55	1.95	
4	3	10	0.5	0	36428	21.31	2.13	
4	3	10	0.5	0	40257	24.55	2.45	
4	4	10	2.0	0	39525	23.93	2.39	2.22
4	4	10	2.0	0	39221	23.67	2.36	
4	4	10	2.0	0	34936	20.04	2.00	
4	4	10	2.0	0	36545	21.41	2.14	
4	5	10	0	0.06	35459	20.49	2.04	2.17
4	5	10	0	0.06	36826	21.64	2.16	
4	5	10	0	0.06	43863	27.60	2.76	
4	5	10	0	0.06	31431	17.08	1.70	

4	6	10	0.5	0.06	36341	21.23	2.12	1.33
4	6	10	0.5	0.06	23654	10.48	1.04	
4	6	10	0.5	0.06	24696	11.37	1.13	
4	6	10	0.5	0.06	23538	10.39	1.03	
4	7	10	2.0	0.06	23311	10.20	1.02	1.39
4	7	10	2.0	0.06	35735	20.72	2.07	
4	7	10	2.0	0.06	28262	14.39	1.43	
4	7	10	2.0	0.06	23502	10.36	1.03	
4	8	10	0	0.6	32354	17.86	1.78	1.95
4	8	10	0	0.6	34128	19.36	1.93	
4	8	10	0	0.6	36540	21.40	2.14	
4	8	10	0	0.6				
4	9	10	0.5	0.6	33592	18.91	1.89	1.82
4	9	10	0.5	0.6	37570	22.28	2.22	
4	9	10	0.5	0.6	27910	14.09	1.40	
4	9	10	0.5	0.6	32223	17.75	1.77	
4	10	10	2.0	0.6	23307	10.19	1.01	0.98
4	10	10	2.0	0.6	22971	9.91	0.99	
4	10	10	2.0	0.6	22621	9.61	0.96	
4	10	10	2.0	0.6	22696	9.68	0.96	
5	1	0	0	0	42229	26.22	2.62	2.65
5	1	0	0	0	41955	25.99	2.59	
5	1	0	0	0	43071	26.93	2.69	
5	1	0	0	0	43129	26.98	2.69	
5	2	10	0	0	45898	29.33	2.93	2.67
5	2	10	0	0	48371	31.42	3.14	
5	2	10	0	0	37415	22.14	2.21	
5	2	10	0	0	39774	24.14	2.41	
5	3	10	0.5	0	31395	17.05	1.70	2.00
5	3	10	0.5	0	38267	22.87	2.28	
5	3	10	0.5	0	35118	20.20	2.02	
5	3	10	0.5	0	35045	20.14	2.01	
5	4	10	2.0	0	43316	27.14	2.71	2.43
5	4	10	2.0	0	38353	22.94	2.29	
5	4	10	2.0	0	35117	20.20	2.02	
5	4	10	2.0	0	43398	27.21	2.72	
5	5	10	0	0.06	39069	23.54	2.35	2.23
5	5	10	0	0.06	35775	20.75	2.07	
5	5	10	0	0.06	38291	22.89	2.28	
5	5	10	0	0.06	37390	22.12	2.21	
5	6	10	0.5	0.06	39224	23.68	2.36	2.17
5	6	10	0.5	0.06	35540	21.40	2.14	
5	6	10	0.5	0.06	33622	18.93	1.89	
5	6	10	0.5	0.06	38358	22.94	2.29	
5	7	10	2.0	0.06	35724	20.71	2.07	2.11
5	7	10	2.0	0.06	36671	21.51	2.15	

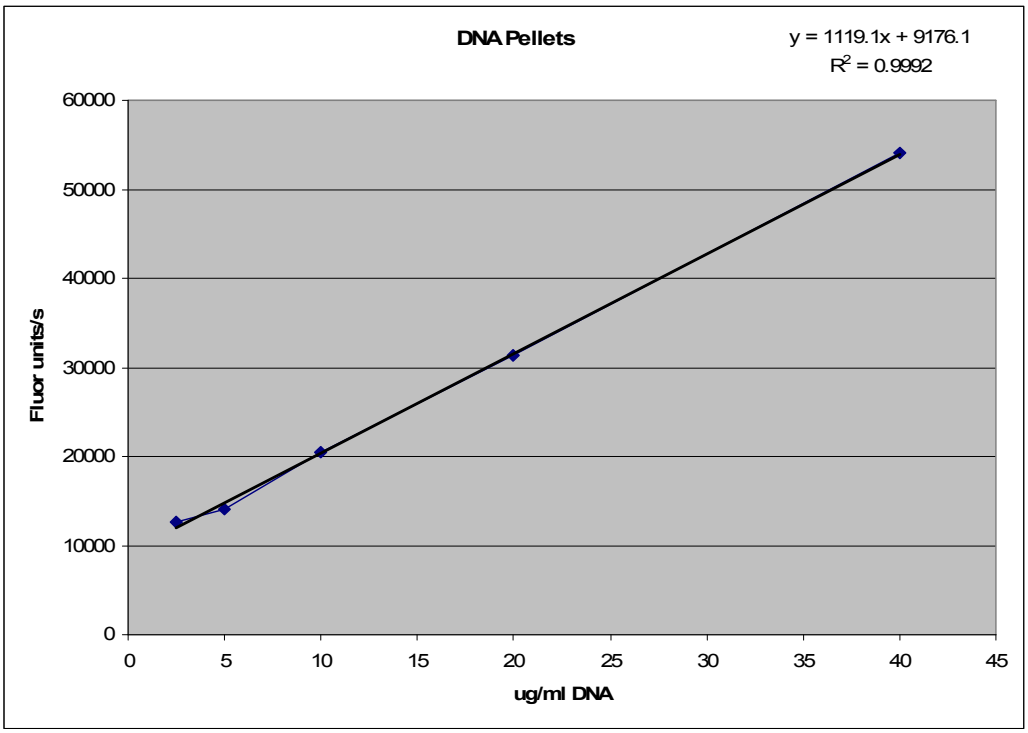
5	7	10	2.0	0.06	32877	18.30	1.83	
5	7	10	2.0	0.06	39703	24.08	2.40	
5	8	10	0	0.6	37876	22.53	2.25	2.25
5	8	10	0	0.6	35631	20.63	2.06	
5	8	10	0	0.6	39029	23.51	2.35	
5	8	10	0	0.6	39126	23.59	2.35	
5	9	10	0.5	0.6	36241	21.15	2.11	2.24
5	9	10	0.5	0.6	38185	22.80	2.28	
5	9	10	0.5	0.6	36866	21.63	2.16	
5	9	10	0.5	0.6	39664	24.05	2.40	
5	10	10	2.0	0.6	34787	19.92	1.99	1.90
5	10	10	2.0	0.6	30486	16.28	1.62	
5	10	10	2.0	0.6	34968	20.07	2.00	
5	10	10	2.0	0.6	34666	19.82	1.98	
6	1	0	0	0	10425	1.68	0.16	0.24
6	1	0	0	0	12413	2.92	0.29	
6	1	0	0	0	12312	2.85	0.28	
6	1	0	0	0	11302	2.23	0.22	
6	2	10	0	0	12022	2.67	0.26	0.38
6	2	10	0	0	12852	3.19	0.31	
6	2	10	0	0	17186	5.88	0.58	
6	2	10	0	0	13524	3.60	0.36	
6	3	10	0.5	0	11313	2.23	0.22	0.19
6	3	10	0.5	0	9976	1.40	0.14	
6	3	10	0.5	0	11208	2.17	0.21	
6	3	10	0.5	0	11172	2.15	0.21	
6	4	10	2.0	0	12045	2.69	0.26	0.31
6	4	10	2.0	0	12016	2.67	0.26	
6	4	10	2.0	0	13603	3.65	0.36	
6	4	10	2.0	0	13357	3.50	0.35	
6	5	10	0	0.06	13077	3.33	0.33	0.27
6	5	10	0	0.06	12799	3.15	0.31	
6	5	10	0	0.06	10863	1.95	0.19	
6	5	10	0	0.06	11511	2.36	0.23	
6	6	10	0.5	0.06	10963	2.02	0.20	0.23
6	6	10	0.5	0.06	11620	2.42	0.24	
6	6	10	0.5	0.06	11496	2.35	0.23	
6	6	10	0.5	0.06	11821	2.55	0.25	
6	7	10	2.0	0.06	10263	1.58	0.15	0.23
6	7	10	2.0	0.06	12533	2.99	0.29	
6	7	10	2.0	0.06	11056	2.07	0.20	
6	7	10	2.0	0.06	12271	2.83	0.28	
6	8	10	0	0.6	11525	2.36	0.23	0.21
6	8	10	0	0.6	11110	2.11	0.21	
6	8	10	0	0.6				
6	8	10	0	0.6	10944	2.00	0.20	

6	9	10	0.5	0.6	12447	2.94	0.29	0.23
6	9	10	0.5	0.6	12352	2.88	0.28	
6	9	10	0.5	0.6	9595	1.17	0.11	
6	9	10	0.5	0.6	11691	2.47	0.24	
6	10	10	2.0	0.6	10094	1.48	0.14	0.21
6	10	10	2.0	0.6	12043	2.69	0.26	
6	10	10	2.0	0.6	11238	2.19	0.21	
6	10	10	2.0	0.6	11627	2.43	0.24	
7	1	0	0	0	9306	1.63	0.16	0.18
7	1	0	0	0	10828	2.79	0.27	
7	1	0	0	0	9222	1.57	0.15	
7	1	0	0	0	9025	1.42	0.14	
7	2	10	0	0	10814	2.78	0.27	0.30
7	2	10	0	0	12329	3.93	0.39	
7	2	10	0	0	11503	3.30	0.33	
7	2	10	0	0	10106	2.24	0.22	
7	3	10	0.5	0	10591	2.61	0.26	0.22
7	3	10	0.5	0	10254	2.35	0.23	
7	3	10	0.5	0	9020	1.41	0.14	
7	3	10	0.5	0	10843	2.80	0.28	
7	4	10	2.0	0	10887	2.83	0.28	0.20
7	4	10	2.0	0	10773	2.74	0.27	
7	4	10	2.0	0	9692	1.92	0.19	
7	4	10	2.0	0	8067	0.69	0.06	
7	5	10	0	0.06	7755			0.13
7	5	10	0	0.06	8142	0.75	0.07	
7	5	10	0	0.06	9658	1.90	0.19	
7	5	10	0	0.06	7438			
7	6	10	0.5	0.06	8221	0.81	0.08	0.14
7	6	10	0.5	0.06	9874	2.06	0.20	
7	6	10	0.5	0.06	10158	2.28	0.22	
7	6	10	0.5	0.06	8221	0.81	0.08	
7	7	10	2.0	0.06	11233	3.09	0.30	0.28
7	7	10	2.0	0.06	10065	2.21	0.22	
7	7	10	2.0	0.06	11278	3.13	0.31	
7	7	10	2.0	0.06	11152	3.03	0.30	
7	8	10	0	0.6	11189	3.06	0.30	0.25
7	8	10	0	0.6	10729	2.71	0.27	
7	8	10	0	0.6	9451	1.74	0.17	
7	8	10	0	0.6	6495			
7	9	10	0.5	0.6	10752	2.73	0.27	0.27
7	9	10	0.5	0.6	11174	3.05	0.30	
7	9	10	0.5	0.6	12506	4.06	0.40	
7	9	10	0.5	0.6	8416	0.96	0.09	
7	10	10	2.0	0.6	8431	0.97	0.09	0.18
7	10	10	2.0	0.6	10624	2.63	0.26	

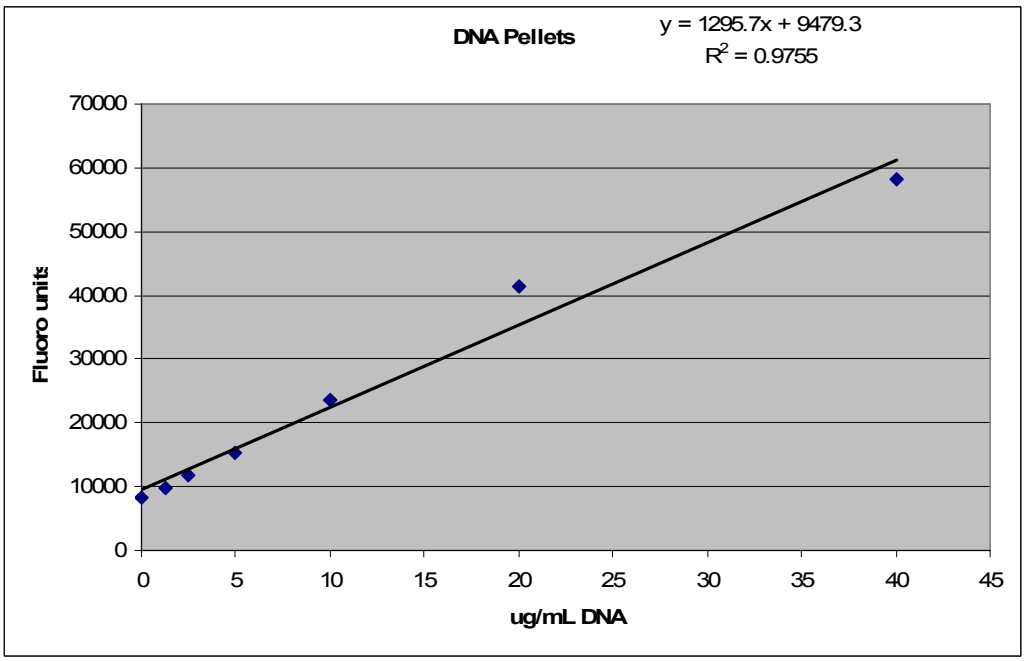
7	10	10	2.0	0.6	9150	1.51	0.15
7	10	10	2.0	0.6	10238	2.34	0.23

Standard curves for DNA analysis:

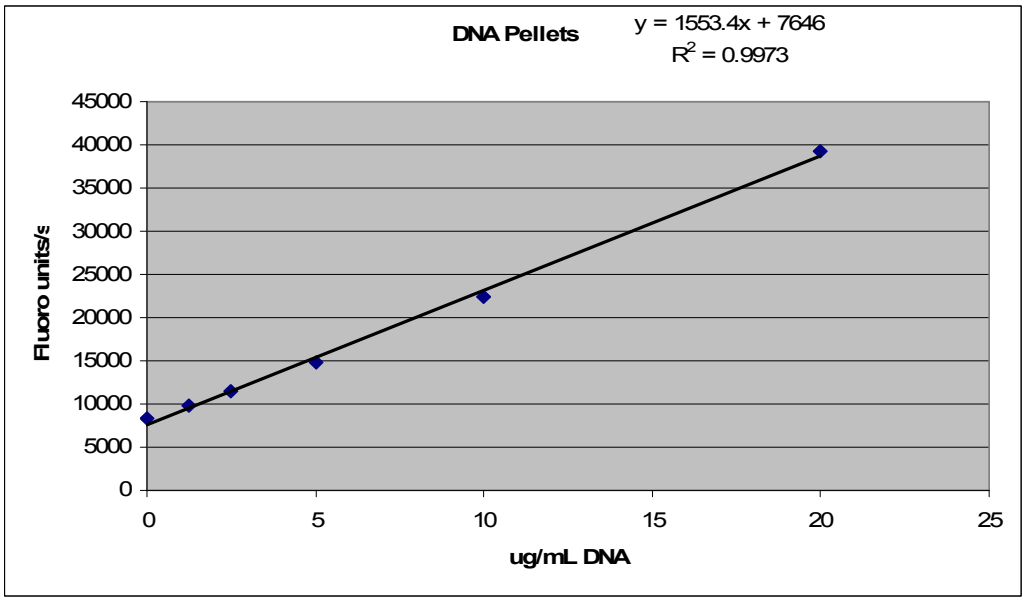
Horse 1



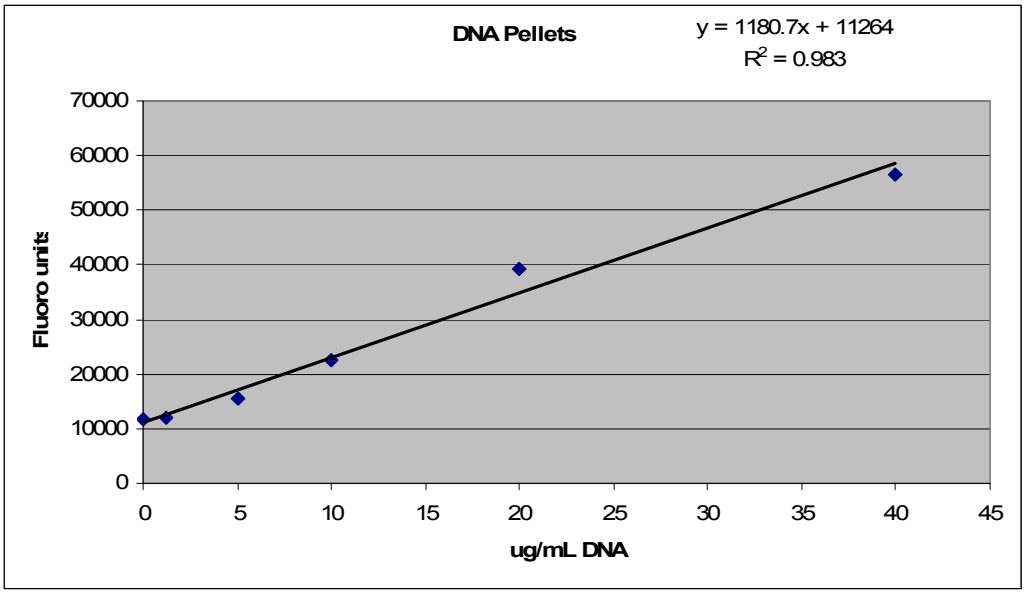
Horse 2



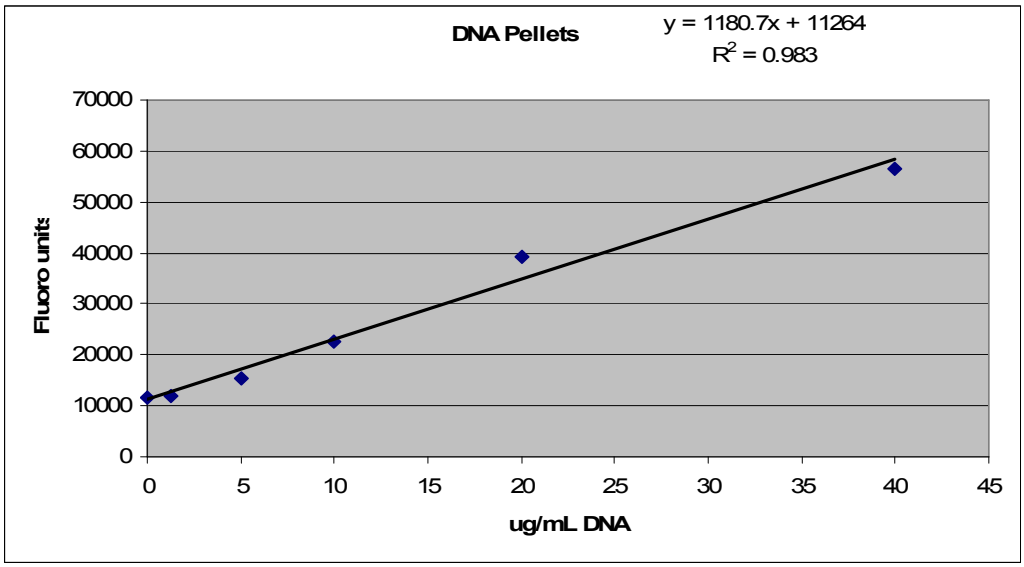
Horse 3



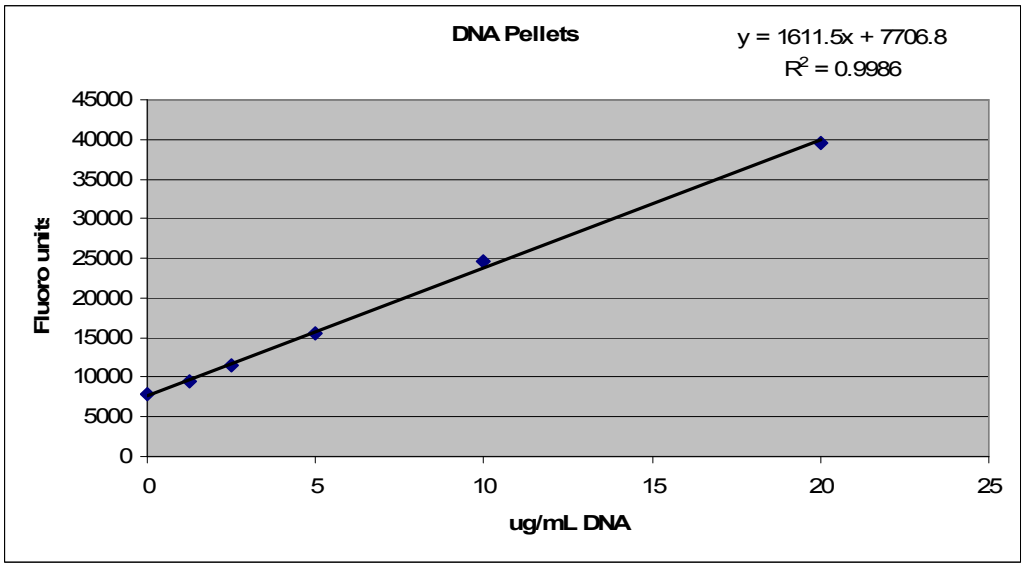
Horse 4



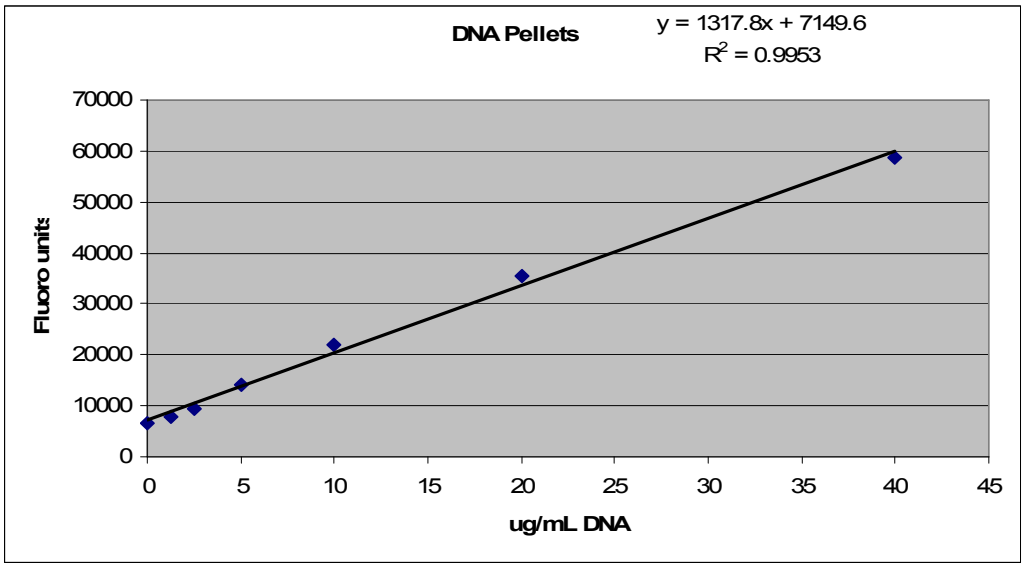
Horse 5



Horse 6



Horse 7


DNA Pellets
y = 1317.8x + 7149.6
R2 = 0.9953
Fluoro units
70000
60000
50000
40000
30000
20000
10000
0
0
5
10
15
20
25
30
35
40
45
ug/mL DNA